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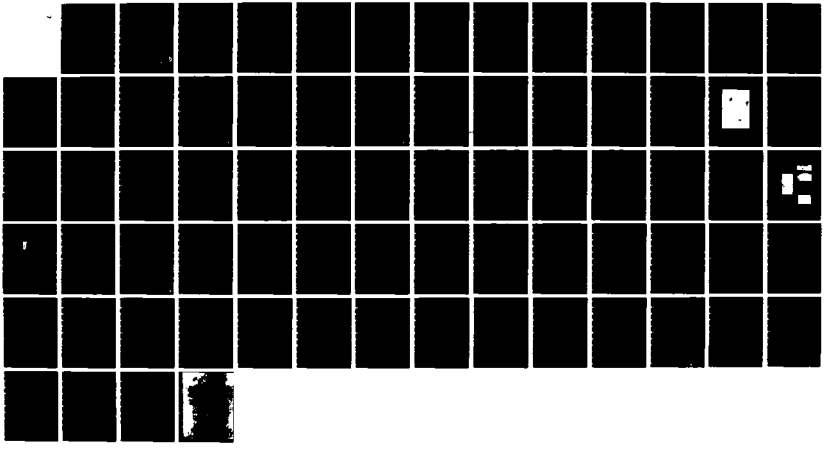

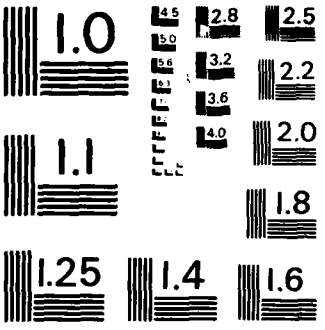

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Report F49620-80-C-0085

CHEMICAL CARCINOGEN (HYDRAZINE et al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID FIBROBLASTS IN VITRO

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23 May 1984

Final Report for Period 1 July 1980 - 30 November 1983

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The initiation of a carcinogenic insult appears to be optimally effective when the cells are insulted in early S phase of the cell cycle. The transformed phenotype has sarcoma associated determinants that are similar to the ectopic determinants found on human sarcoma-tumor tissue. Both these cell types, i.e. chemically transformed human fibroblasts and sarcoma tumor cells, exhibit cellular invasiveness, neoplastic potential and a finite but extended lifespan in vitro. Using selection pressures for culturing of human normal cells in culture followed by insulting the DNA we can isolate cells from the transformed

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phenotype, from cells that will respond to the insult as a toxic insult. The carcinogen-specific DNA adducts formed under these conditions are qualitatively and quantitatively similar.

Final Technical Report

CHEMICAL CARCINOGEN (HYDRAZINE et al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID FIBROBLASTS IN VITRO

Dates of Report: July 1, 1980 to November 30, 1983

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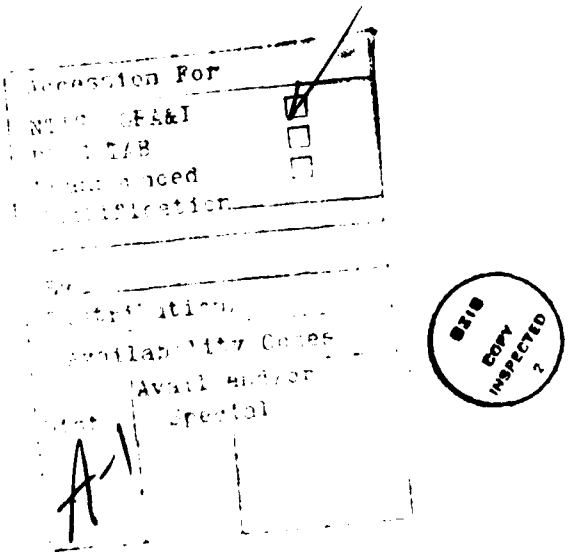

Animal Use Statement

The experiments reported herein were conducted according to the principles described in "Guide for the Care and Use of Laboratory Animals" prepared for the Committee on Care and Use of Laboratory Animals, DHHS Publication No. NIH 78-23, revised 1982.

George E. Milo

George E. Milo, Ph.D.
Comprehensive Cancer Center

The Ohio State University has on file with the Office of Protection from Research Risks, NIH, a statement of assurance concerning the care and treatment of laboratory animals. This assurance states that the University complied with NIH Guide for the Care and Use of Laboratory Animals, applicable portions of Public Law 91-579, and related rules and regulations issued by the Secretary of Agriculture.

Richard L. Wright
Deputy Director for Development
The Ohio State University
Research Foundation

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A final report on contract no. F49620-77-0110 was finalized in 1979. The work following that period under contract F49620-80-C-0085 was a continuation of this effort with a new work scope.

Work Scope 1980-1981

a) To investigate how UDMH, HZ and/or BP are transported to the cell nucleus.

b) To investigate changes in DNA directed DNA polymerase I and II activity following treatment with UDMH, HZ and/or BP.

c) To examine changes in histone labeling patterns during the early and late stages of the carcinogenic process following exposure to UDMH or BP.

d) To investigate the interaction of HZ and BP metabolites with DNA during the induction process and correlate this with the metabolic profiles in the activation stage.

e) To develop a predictable and reliable procedure using human cells in vitro to evaluate the carcinogenic potential of chemicals of interest to the Air Force.

Abstract

There is data from in vivo animal systems that DMH and polynuclear hydrocarbons may pose a potential carcinogenic risk to man. They are metabolized to reactive intermediate metabolites that are localized in suspectible target sites. These sites may be away from the tissue that activates the proximate carcinogen. One such highly reactive intermediate obtained from DMH metabolism is methylazoxy methanol. This compound purportedly degrades to form methyldiimine and formaldehyde. Methyldiimine then forms a methyl radical after homolysis. This compound then is converted to a carbonium ion and the radical interacts with the purine bases in DNA. Methylazoxymethanol acetate, (MAMA) in the presence of colon, secum and liver homogenates reduced NAD^+ to NADH. These "alcohol dehydrogenase-"-like enzymes are quite high in activity in the liver and may account for the organotypic response of MAM in animals.

We completed biochemical studies to examine how these carcinogens were activated, entered the human cell and were transported to the nucleus. We also studied how these reactive carcinogenic intermediates interacted with different bases in the DNA.

Conclusions: 1980-1981

1. BP was transported to the nucleus via a lipoprotein complex where it was activated to an oxygenated form. The 7R-BPDEI-(+) anti form of BP interacted with guanine to form the proper adduct to induce a carcinogenic response.

2. The hydrazine compounds, 1,1- and 1,2-dimethyl forms of HZ alkylated directly the DNA, namely, N^7 of guanine and O^6-guanine. Again using a compound not requiring activation the appropriate DNA-adducts were formed.

3. The metabolic profiles of BP metabolism by the plasma membrane associated P_{450} oxygenase enzymes indicated that this activation process was primarily associated with producing toxic metabolites that resulted in cellular toxicity rather that a carcinogenic respose.

4. There were no changes in α, β or γ DNA polymerase activity of cells treated with a carcinogenic non-toxic dose of BP or 1,1-; 1,2-DMH. Furthermore, semi-conservative DNA synthesis pattern of synchronous cells in S was not altered. The profile of S was determined by autoradiography following the incorporation of [^{3}H-CH_3]-thymidine into DNA.

5. We detected no change in methylation, acetylation or phosphorylation of histone proteins as reported for rodent cell systems at the time of initiation. At the time of expression of the transformed phenotype 10 PDL following the conclusion of treatment, we did at that time see a change in labeling patterns of histones primarily in the H_1 histone.

6. We found that FeSV transformation and U.V.$_{254}$ nm induction of transformation followed a similar pattern of induction of transformation, i.e. in a narrow window of time in early S the cells were optimally responsive to the carcinogenic insult.

Papers Published 1980-1981

1. Milo, G., R. Olsen, S. Weisbrode, and J. McCloskey (1980) Feline sarcoma virus induced in vitro progression from premalignant to neoplastic transformation of human diploid cells. In Vitro. 16: 813-822.

2. Milo, G., and J. DiPaolo (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Int. J. Cancer. 26: 805-812.

3. Milo, G., A. Aackerman, and I. Noyes (1980) Growth and ultrastructural characterization of proliferating keratinocytes in vitro without added extrinsic factors. In Vitro 16: 20-30.

4. Tejwani, R., S. Nesnow, and G. Milo (1980) Analysis of intracellular distribution and binding of benzo(a)pyrene in human diploid fibroblasts. Cancer Letters 10: 57-65.

5. Milo, G., and J. DiPaolo (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Int. J. of Cancer. 26: 805-812.

6. Milo, G., S. Weisbrode, R. Zimmerman, and J. McCloskey (1981) Ultraviolet radiation induced neoplastic transformation of normal human cells. Chem. Biol. Int.36: 45-59.

7. Milo, G., R. Trewyn, R. Tejwani, and J. Oldham (1981) Intertissue variation in benzo(a)pyrene metabolism by human skin, lung and liver, in vitro. Advisory group for aerospace research and development. Sci. Tech. Aerospace Rep. 18: 2533-2541.

Work Scope 1981-1982

a) To examine changes in histone labeling patterns or carcinogen-nuclear non-histone protein binding during the early and late stages of the carcinogenic process following exposure to different carcinogens.

b) To investigate the interaction of BP and/or BP metabolites with DNA during the induction process and correlate these adducts with BP oxygenated metabolites produced during the activation stage.

c) To investigate different nitrosamines for their carcinogenic activities.

d) To investigate the role of promoters (modulators) in the induction process.

e) To study the interaction of BP and/or BPDE-I with cellular DNA of responsive and refractory cell populations.

f) To investigate the role of the cell cycle in influencing chemical induction of human cell transformation.

g) To investigate the effects of hydrazines and their analogues on unscheduled DNA synthesis.

Abstract

Polynuclear hydrocarbons or hydrazine analogues do not have an absolute requirement for binding to DNA in order to elicit a carcinogenic event. Furthermore, PNH like BP enter the nucleus via a lipoprotein complex where the BP is oxygenated and then interacts with the genetic material, (DNA). The adduct formed is 7β-BPDEI-dG. Benzamide does not alter the binding of the perceived ultimate carcinogen to dG but eliminates the carcinogenic response. The period of heightened response to the carcinogen appears to be 3 hrs into S phase of the cell type. The modifying effect of TPA or benzamide appears to alter the nuclear non-histone proteins protecting the cellular DNA.

Conclusion: 1981-1982

The specific points addressed in the previous years' conclusions were then examined again in the presence of benzamide (BZ), an inhibitor of the carcinogenesis process.

1. It was found that the BZ compound, while inhibiting the expression of carcinogenesis, did not alter the profile of specific carcinogen-DNA adduct formation.

2. Unscheduled DNA synthesis in the presence of and/or absence of BZ was not altered. Moreover, when an insult was delivered to the cells in G_1 part of the cell cycle compared to S there was no difference in repair of the lesions in DNA. However, cells treated in G_1 exhibited no carcinogenic response to the insult while cells treated in S did exhibit a carcinogenic response.

3. Human foreskin fibroblast populations blocked in G_1, released and treated with methylazoxy methanol acetate (MAMA) from the time of release (late G_1) for 1 hr treatment intervals until 4 hrs into S, exhibited a differential sensitivity to MAMA treatment at the different treatment times. A heightened response to the carcinogen treatment was not detected until calmodulin, a cell regulatory protein, was optimally present in the nuclei of the late G_1 treated cells 6 hrs after release from the G_1 block. Moreover, there was a distinct increase in the number of transformed phenotypes, (cells that will grow in soft agar) observed when the cells were treated with MAMA at the onset of scheduled DNA synthesis. The time at which these treated cells were optimally responsive to a carcinogenic insult was 12-13 hrs after release from the block 2-3 hrs into S. Interestingly, this was followed by a decrease in the expression of anchorage independent growth when the cells were treated 13-14 hrs after release from the block 4 hrs into S. Benzamide interfered in the process when added at the onset of S and the resultant carcinogen treated population did not exhibit a comparable increase in expression of anchorage independent growth. Cells treated with MAMA at the point of release from the block G_1^{0-1} to G_1^{3-4} did not express anchorage independent growth.

4. Cell cycle studies to study the effects of phorbol esters on cell cycle activation using the published cytofluorometric procedures developed for animal cell systems were not translatable to human cells. Dr. D. Tomei had to abandon the published techniques designed for animal studies and reevaluate the application of published systems to human cell kinetic cell cycle work. The vital stains used to stain cellular DNA would not work. We are continuing to evaluate these stains as described above. Either scheduled DNA synthesis in human cells undergoes a multiple initiation point of the beginning of DNA synthesis or the intercalating DNA dyes do not penetrate the nucleus in the same manner as that described for animal cells. By modifying our isolation procedures we now are obtaining yields in excess of 50% clean nuclei. The application of these techniques permitted us to investigate the toxic effects of compounds on the nuclei of the layers of skin grown in vitro.

Papers Published 1981-1982

1. Tejwani, R., R. Trewyn, and G. Milo. (1981) Kinetics of movement of benzo(a)pyrene into transformable and nontransformable diploid fibroblasts. Symposium on Polynuclear Aromatic Hydrocarbons, Fifth International Symposium, Battelle Mem. Inst. 5:97-107.

2. Cazer, F., M. Inbasekaran, J. Loper, R. Tejwani, D. Witiak and G. Milo (1981). Human cell neoplastic transformation with benzo(a)pyrene and a bay region reduced analogue of 7,12-dimethylbenz(a)anthracene. Symposium on Polynuclear Aromatic Hydrocarbons, Fifth International Symposium, Battelle Mem. Inst. 5:499-507.

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6. Milo, G., and R. Trewyn (1982) In vitro transformation of cultured human diploid fibroblasts. Banbury Report. Nitrosamines and Human Cancer. Edited by Peter N. Magee. Cold Spring Harbor Laboratory. 12: 3-13.

Work Scope 1982-November 30,1983

a) To develop a procedure that is both predictable and reliable, using human cells in vitro to evaluate the potential carcinogenic activity of suspect carcinogens.

b) To study the effect promoters and sensitizers have on the preinduction process.

c) To study the interrelationships between analogues of carcinogens and their carcinogen activity.

d) To investigate the role of the cell cycle in influencing chemical induction of human cell transformation.

e) To continue studies directed towards understanding the role of the carcinogens evaluated in 1981-1982 scope of work.

Summary

We have developed a reliable and reproducible biological endpoint to evaluate the carcinogenic endpoint that extends beyond the work published in IN VITRO 17: 719-729, 1981. We can evaluate the endpoint for an expression of cellular invasiveness on chick embryonic skin in vitro. We can evaluate the neoplastic potential of the transformed cells upon injection of a bolus of transformed human cells intracranially into the frontal sinus of a nude mouse. We can reliably and reproducibly evaluate the transformed cells for the presence of a sarcoma associated determinant using a monoclonal antibody directed against the cell surface determinant. We have tissue typed a range of sarcoma tumor tissues from humans and found one case that was negative in > 25 tumors evaluated to date.

Secondly, we have examined each phase of the cell cycle for its responsiveness to a carcinogenic insult. Human foreskin fibroblast populations blocked in G_1, released, and treated with methylazoxymethanol acetate (MAMA) from the time of release (late G_1) for 1 hr treatment intervals until 4 hrs into S exhibited a differential sensitivity to MAMA treatment at the different treatment times. A heightened response to the carcinogen treatment was not detected until calmodulin, a cell-regulatory protein, was optimally present in the nuclei of the late G_1-treated cells 6 hrs after release from the G_1 block. Moreover, there was a distinct increase in the number of transformed phenotypes (cells that will grow in soft agar), observed when the cells were treated with MAMA at the onset of scheduled DNA synthesis. The time at which these treated cells were optimally responsive to a carcinogenic insult was 12-13 hrs after release from the block 2-3

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It was proposed that the heightened presence of calmodulin in the nuclei 4 hrs prior to the onset of scheduled DNA synthesis is a cell-regulatory function that sets in motion a complex series of events (program) in carcinogen-initiated human fibroblasts that leads to a subsequent carcinogenic response.

The specific DNA-carcinogen adducts formed under these conditions were qualitatively and quantitatively no different in non BZ-carcinogen treated cells when compared to BZ-carcinogen treated cells. We conclude from these studies that specific adduct formation between the carcinogen and bases in the DNA does not appear to be the critical mechanism for induction of a transformed human phenotype. We recognized that the toxic insult plays a role in the program of cellular events that leads to a carcinogenic response but is not necessarily a requirement when the events leading to an initial expression of carcinogenesis are not properly programmed into the dynamic cell cycle phase.

In the narrow window of time where the cell in S is responsive to the carcinogenic insult (Milo, G. (1983) In vitro transformation of human cells: modulation of early gene expression preceding carcinogen induced events. Human Carcinogenesis. Eds. Harris and Autrup, Chapt. 17: 431-449. Academic Press, N.Y.) we do not know whether the carcinogen-adducts formed in early S are critically formed in the fast replicating DNA or the parental strand of DNA. We are at the present engaged in the following experiments to evaluate the above:

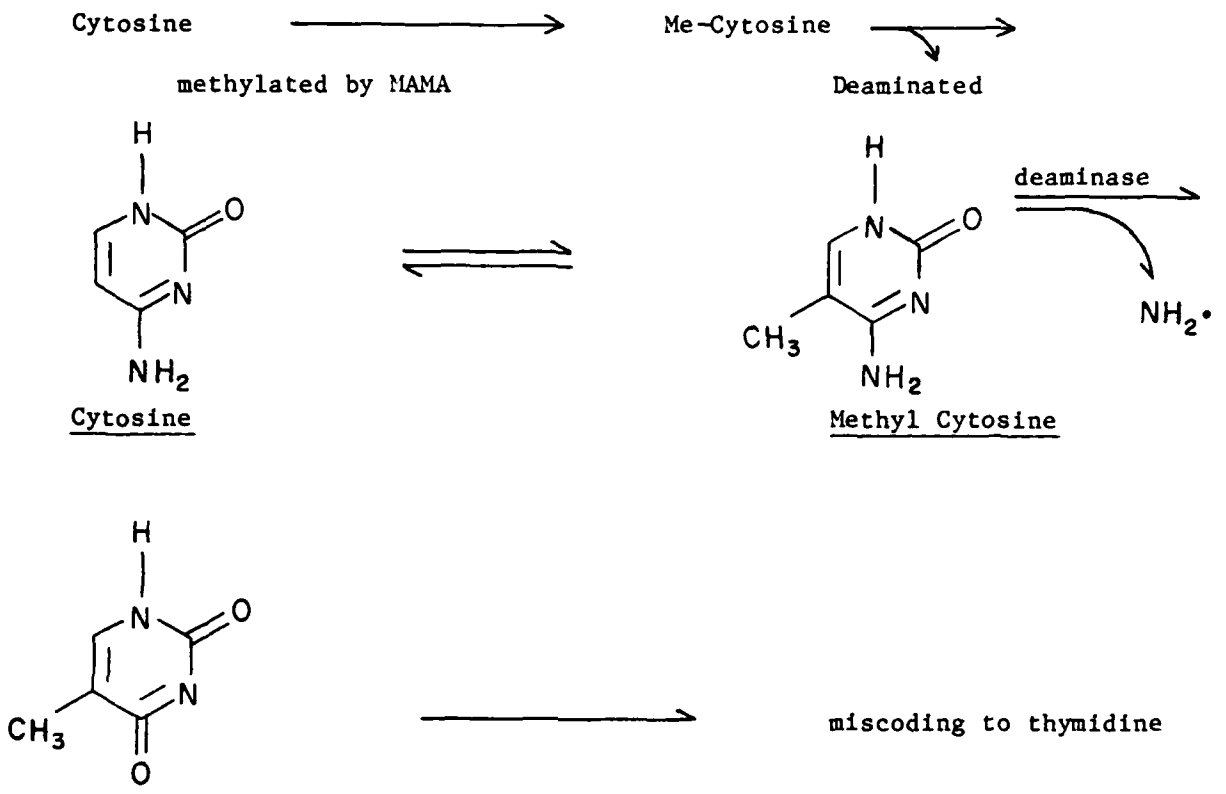
1. Experiments to look at specific carcinogen-DNA adducts in fast replicating and parental DNA in the presence of BZ.

2. Using either [^{3}H]-G or [^{14}C]-G labeled Budr we will examine the isolated Budr labled DNA in the light and heavy strands of DNA to see if there is preferential binding of the carcinogen in the presence or absence of BZ.

3. We are developing post $^{32}P_i$ labeling technology to measure carcinogen-DNA adducts at the fentamole (10^{-15}moles) level rather than the micromole (10^{-6}moles) level. This technology will permit us to increase our level of sensitivity for detection of specific carcinogen minor modification of the DNA and quantitate minor modifications in the DNA. The addition of BZ to the carcinogen treated cultures will permit us to examine how the cells will respond to a toxic insult. Another biological response the treated cells may express is a mutagenic response. Under these conditions, when grown on 6-thioguanine supplemented medium, the cells produce mutants. These hprt mutants were amplified when the cells were treated in late G_1 4 hrs prior to the onset of S. In the presence of BZ, added at the G_1 release point for 10 hrs and removed at the onset of S, transformants were reduced to zero while not affecting the expression of the point mutations, (hprt mutants). We can, therefore, by selecting when we add the carcinogen, either elicit a mutagenic response, a carcinogenic response, or a toxic response. We suspect, due to the short period of time that elapses between the initial insult and the fixation of the insult that the initial insult is fixed in the cell in the following manner:

FIGURE 1

Genetic Coding Mechanism



Phenotype

G≡ C

↓ methylation during S

G≡ C-Me

↓

C≡ G Uracil-Me
replicating DNA

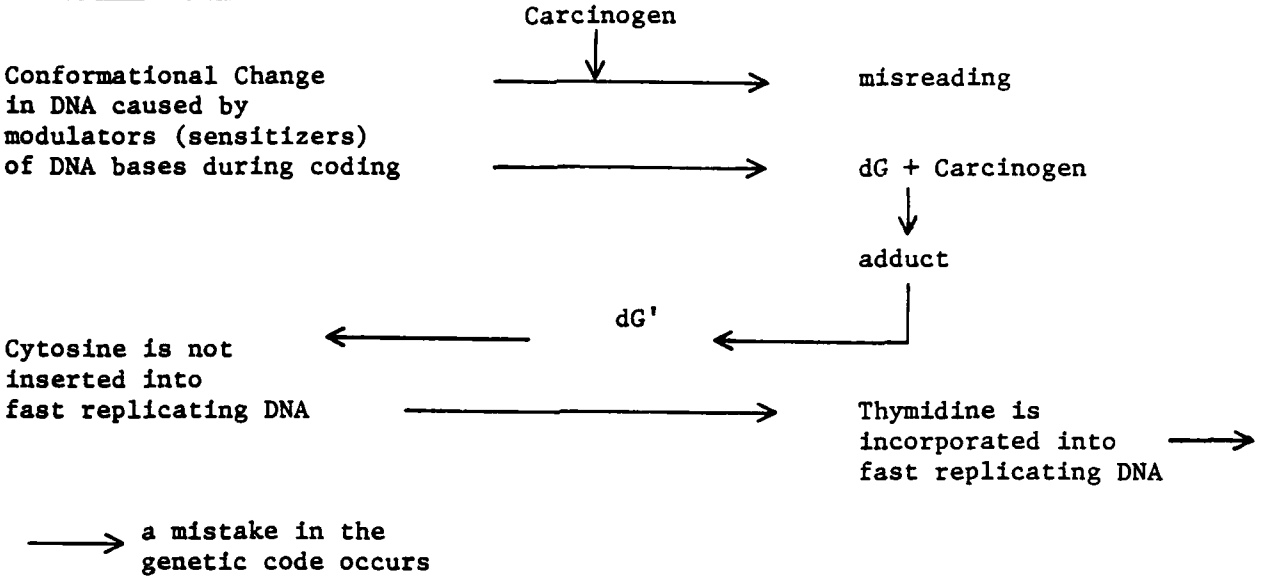
parental DNA → daughter cell
Uracil codes with T

Reading frame shifts

FIGURE 2

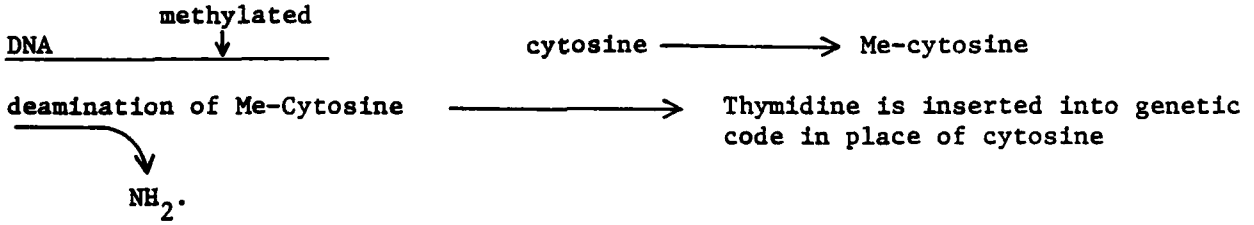
Proposal: Molecular Mechanisms to Explain Genotoxic Insults

1. Bulky Adducts



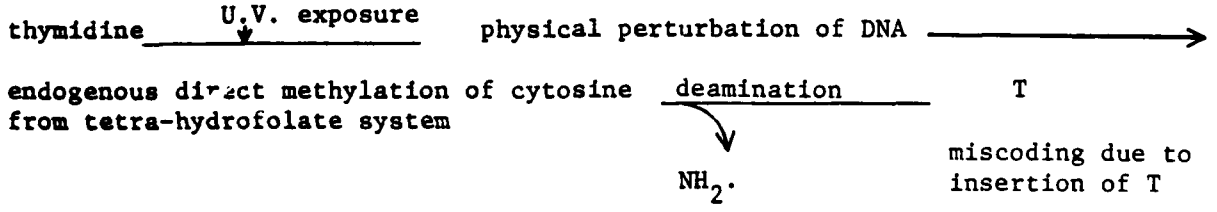
2. Non-bulky adducts

These are formed by akylating agents such as 1, 1- or 1, 2-UDMH



3. Physical Carcinogen

U.V. transformation



We will study these proposed pathways by examining the treated cells for deaminase activity, incorporation of radiolabeled methionine into BZ and non-BZ treated cells, etc.

Publications 1982-1983

The work on the inhibitors of the carcinogenesis process in collaboration with Dr. E. Kun was forwarded to AFOSR last fall in an annual report. This work was not reported on under this cover. Furthermore, the collaborative work with Dr. Donald Witiak is presently being written into three manuscripts, one which is included under this cover, and two in preparation.

1. Howard, P., J. Gerard, G. Milo, P. Fu, F. Beland and F. Kadlubar (1983) Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo(a)pyrene. Carcinognensis 4: 353-355.

2. Milo, G. (1983) In vitro transformation of human cells: modulation of early gene expresison preceding carcinogen induced events. Human Carcinogenesis. Edited by Curtis Harris and Herman Autrup. Chapt. 17: 431-449. Academic Press, N.Y.

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4. Kun, E., T. Miraga, E. Kirsten, G. Jackowski, J. McLick, L. Peller, S. Oredsson, L. Martin, N. Pattabiraman, and G. Milo (1983) Biochemical basis of the regulatory role of polyadenosine diphosphoribose. Adv. in Enz. Reg. 21: 177-197.

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6. Kumari, L., P. Kamat, S. D'Ambrosio, D. Witiak, and G. Milo (1983) DNA modification and damage by dimethylhydrazine and methylazoxymethanol acetate. Neoplastic transformation and cytotoxicity of human diploid cells. Cancer Res. in preparation.

7. Kumari, L., D. Witiak and G. Milo (1983) Effect of anticarcinogen, benzamide on molecular perturbation of DNA by MAMA. Transformation of human cells. Carcinogenesis in preparation.

Carcinogenesis Vol.4 No.3 pp.353 – 355, 1983

Short Communication

Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo[a]pyrene

Paul C.Howard, Jeff A.Gerrard[1], George E.Milo[1], Peter P.Fu, Frederick A.Beland, and Fred F.Kadlubar
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(Received on 18 August 1982; accepted on 28 December 1982)

Abstract

The potent mutagens, 1-nitropyrene and 6-nitrobenzo[a]pyrene, are shown to induce the transformation of cultured normal human diploid fibroblasts to a state of anchorage-independent growth and cellular invasiveness. The transformation frequencies are greatly enhanced by addition of bovine milk xanthine oxidase, a mammalian nitroreductase.

Polycyclic aromatic hydrocarbons (PAH's)* are environmental contaminants which are metabolized in a wide variety of species to reactive electrophiles that bind to cellular constituents and lead to mutations and cancer (1 – 3). Recent evidence has demonstrated that a subclass of these compounds, the nitro-substituted PAH's, are found in fly ash, diesel emissions, photocopier fluids, cigarette smoke, and environmental samples (4 – 14). These nitroarenes are highly mutagenic in microbial test systems (7 – 9, 15 – 17), and appear to be responsible for a large proportion of the mutagenic activity of airborne particulates (10). Nitro-substituted PAH's are also known to be appreciably less mutagenic in nitroreductase-deficient *Salmonella typhimurium* strains than in normal tester strains (8,15 – 17), which suggests that metabolic reduction of the nitro group is an essential step in the mutagenic activation of these compounds.

We have recently reported that bovine milk xanthine oxidase, a mammalian nitroreductase, will reduce the nitro-PAH, 1-nitropyrene, to an electrophilic species that binds covalently to DNA (18); and evidence has been presented which indicates that the N-hydroxy arylamine, N-hydroxy-1-aminopyrene, is the ultimate mutagenic metabolite of 1-nitropyrene (18,19). We have also reported that the carcinogenic N-hydroxy derivatives of 1- and 2-naphthylamine induce the transformation of cultured human fibroblasts to a state of anchorage-independent growth (20). These neoplastic cells were also capable of progressing to tumors in nude mice following implantation.

In the present report, two environmentally occurring nitroarenes, namely 1-nitropyrene and 6-nitrobenzo[a]pyrene, are shown to induce the transformation of cultured normal human diploid fibroblasts to clones that exhibit anchorage-independent growth and cellular invasiveness. In addition, since xanthine oxidase can reduce 1-nitropyrene to a DNA-binding species, cell cultures were supplemented with this enzyme and its substrate, hypoxanthine, in order to determine whether or not exogenous enzymatic nitroreduction would enhance the transformation frequency.

Foreskin-derived normal human diploid fibroblasts (21,22) were seeded at low cell density (23) in Eagle's minimal essential medium prepared with Hank's balanced salt solution (MEM-HBSS) and 20 mM HEPES, pH 7.2, and supplemented with 1x essential amino acids, 1x vitamins, and 20% fetal bovine serum (FBS). Following attachment, the cells were treated under anaerobic conditions with 3, 6, 16 or 33 μM 1-nitropyrene or 4, 13, 34 or 67 μM 6-nitrobenzo[a]pyrene to determine cytotoxicity, as measured by relative cloning efficiency and the ability to grow in soft agar (23).

In the transformation protocol, cells were incubated at high cell density (23) in MEM-HBSS, 20 mM HEPES buffer, pH 7.2, and 10% FBS (maintenance medium) and synchronized to S-phase as described (24,25). The cells were then exposed under anaerobic conditions to either 1-nitropyrene or 6-nitrobenzo[a]pyrene at concentrations that resulted in a 75% relative cloning efficiency at low cell density; i.e., 16 μM for 1-nitropyrene and 34 μM for 6-nitrobenzo[a]pyrene. The absolute plating efficiency for untreated cells was always >95%. When indicated, allopurinol (37 μM) and/or 0.1 U/ml xanthine oxidase along with 3.7 mM hypoxanthine were added. After 2.5 h of incubation, the medium was changed to the maintenance medium and the cells were then incubated for an additional 9.5 h. The cells were then serially passaged 1:2 into 8x selection medium (20,22,24,25), and upon reaching confluence they were passaged five additional times at 1:10 split ratios. These cells were seeded at 5 x 10^4 cells/25 cm^2 into 2 ml of 0.33% agar in supplemented Dulbecco's LoCal medium and layered over a 5-ml base of 2% agar in RPMI-1629 (23). The low calcium conditions employed have been demonstrated to select for transformed phenotypes by inhibiting the growth of normal fibroblasts (26). Colonies of 50 cells or greater were scored 21 days following seeding and the colony-forming ability determined per 10^3 seeded cells.

The cells were then seeded in complete medium (27) and propagated *in vitro* before implantation on chicken embryo skin cultures to determine the invasiveness of the transformed cells (27,28). Embryos were removed from 9 – 10-day-old fertile eggs, the skin was isolated and placed on a 2% agar base containing chicken embryo extract supplemented with MEM-HBSS (26,27). Seeded onto the skins were propagated cells which had been suspended in 25 μl of MEM-HBSS supplemented with 20% FBS. The cells were refed on day 2 with 15 μl of MEM-HBSS supplemented with 20% FBS. The tissues were fixed on day 4 with Bouin's solution, embedded in paraffin, stained with hematoxylin and eosin, and evaluated for invasiveness by light microscopy.

Incubation of normal human fibroblasts at low cell density and under anaerobic conditions with various concentrations of 1-nitropyrene and 6-nitrobenzo[a]pyrene led to a concentration-dependent increase in both cytotoxicity, as in-

*Abbreviations: PAH's, polycyclic aromatic hydrocarbons; MEM-HBSS, Eagle's minimal essential medium with Hank's balanced salt solution; FBS, fetal bovine serum.

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Table I. Relative cloning efficiency and frequency of anchorage-independent growth in soft agar by human diploid fibroblasts exposed to 1-nitropyrene and 6-nitrobenzo[a]pyrene.

Compound	Concentration (μM)	Relative cloning efficiency[a] (%)	Frequency of colony growth in soft agar[b]
1-Nitropyrene	3	100	8 ± 1
	6	90	12 ± 1
	16	75	34 ± 3
	33	50	58 ± 3
6-Nitrobenzo[a]-pyrene	4	100	10 ± 2
	13	90	16 ± 2
	34	75	40 ± 3
	67	50	75 ± 4

[a]Cells were seeded at 40/cm², treated for 2–5 h under anaerobic conditions with varying concentrations of the nitrated PAH, and allowed to grow 7–13 days before determining the relative cloning efficiency. [b]After serial passage in 8x selection medium, 50 000 cells were seeded into 0.33% agar in supplemented Dulbecco's LoCal medium and layered over a 2% agar base in RPMI-1629. The colonies were counted after 21 days and represent the mean ± S.D.

dicated by relative cloning efficiency, and frequency of anchorage-independent growth in soft agar (Table I). Anchorage-independence was not observed under aerobic conditions or after exposure of the cells to the solvent vehicle. 1-Nitropyrene was more cytotoxic than 6-nitrobenzo[a]-pyrene; and, at equal doses, it caused approximately twice as many anchorage-independent colonies as 6-nitrobenzo[a]-pyrene.

The transformation protocol requires that cells be treated at high cell density (23). Under these conditions, nitrated PAH concentrations that gave a 75% relative cloning efficiency in the low cell density cytotoxicity assay (i.e., 16 μM 1-nitropyrene and 34 μM 6-nitrobenzo[a]pyrene) did not elicit any cytotoxicity. These concentrations led to the expression of an average of 14 and 36 transformed colonies/10^5 seeded cells for 1-nitropyrene and 6-nitrobenzo[a]pyrene, respectively (Table II). As before, no transformed colonies were observed under aerobic conditions or after exposure of the cells to the solvent vehicle. Since anaerobic conditions were required during the nitrated PAH exposure period in both the cytotoxicity and transformation experiments, these data suggest that human fibroblasts might metabolize nitroarenes to ultimate carcinogens and that the conversion could involve either an oxygen-sensitive nitroreductase or a reactive metabolite that rapidly decomposed under aerobic conditions.

The addition of hypoxanthine and bovine milk xanthine oxidase, which functions as a nitroreductase under anaerobic conditions (18), resulted in a substantial increase in the transformation frequency to yield an average of 81 and 62 colonies/10^5 cells, respectively, for 1-nitropyrene and 6-nitrobenzo[a]pyrene (Table II). Neither hypoxanthine nor xanthine oxidase were toxic to the cells, nor induced transformation under these conditions. Analysis of the incubation medium by h.p.l.c. indicated a xanthine oxidase-dependent reduction of 1-nitropyrene to 1-aminopyrene. Therefore, exogenous xanthine oxidase appeared to convert 1-nitropyrene and 6-nitrobenzo[a]pyrene to reactive metabolites which entered the fibroblast and induced cellular damage that resulted in a transformed phenotype. Allopurinol, a specific inhibitor of xanthine oxidase (18,29), decreased the transformation frequency for both nitro compounds to ~10 colonies/10^5 cells (Table II). No toxicity was detected in cells treated with allopurinol and h.p.l.c. indicated that this agent completely suppressed xanthine oxidase-catalyzed 1-nitropyrene reduction. For 6-nitrobenzo[a]pyrene, the inhibited level of transformation was significantly lower than that observed with fibroblasts alone and suggests that an endogenous allopurinol-sensitive nitroreductase(s) may be present in the cultured cells.

Table II. Human diploid fibroblast expression of anchorage-independent growth in soft agar and cellular invasiveness on chick embryonic cells following exposure to nitro-substituted PAH's.

Incubation conditions	Experiment	Frequency of colony growth in soft agar[b]	Cellular invasiveness[c]
1-Nitropyrene			
Complete[d]	1	14 ± 5	+
	2	14 ± 7	+
Complete + air	1	0	N.T.
Complete + hypoxanthine/ xanthine oxidase	1	84 ± 14	+
	2	78 ± 1	+
Complete + allopurinol + hypoxanthine/xanthine oxidase	1	9 ± 3	N.T.
	2	10 ± 4	N.T.
Control	1	0[e]	N.T.
	2	0	N.T.
6-Nitrobenzo[a]pyrene			
Complete	1	36 ± 8	+
	2	36 ± 10	+
	3	35 ± 7	N.T.
Complete + air	1	0	N.T.
Complete + hypoxanthine/ xanthine oxidase	1	58 ± 5	+
	2	66 ± 11	+
	3	69 ± 5	N.T.
Complete + allopurinol + hypoxanthine/xanthine oxidase	1	14 ± 5	N.T.
	2	6 ± 3	N.T.
Control	1	1 ± 1	N.T.
	2	0	N.T.

[a]The complete incubation consisted of exposure of the cells for 2.5 h in MEM-HBSS under anaerobic conditions to either 1-nitropyrene (16 μM) or 6-nitrobenzo[a]pyrene (34 μM). When indicated, allopurinol (37 μM) and/or 0.1 U/ml xanthine oxidase and 3.7 mM hypoxanthine were added. Control conditions were the exposure to MEM-HBSS, anaerobically. [b]The cells were prepared, treated, and assayed for growth efficiency in soft agar as described in the text. The frequency indicated is the number of colonies of 50 cells or greater in size, scored 21 days following seeding and expressed as colonies/10^5 seeded cells. Each value is the mean value for three readings/well, with three wells/treatment and is presented as the mean ± S.D. [c]Each of the chick embryonic skin organ cultures received transformed cells as described in the text. The organ cultures were evaluated 3 days later (22). N.T. indicates not tested. [d]Occasionally an aggregate of cells appeared in the agar. These were recognized by the presence of very large cells in the presence of smaller cells in the colony.

A strong correlation exists between tumor formation in nude mice, growth in soft agar, and invasiveness in chicken embryo skin cultures for chemically-transformed cells, or isolated carcinoma cells (27). In this study, all of the cells tested exhibited cellular invasiveness that interrupted 8–13 layers of embryonic skin cells (Table II). Based upon mitotic index and morphological properties, the invasive cell populations have been previously characterized as simulated fibrosarcomas (27).

In conclusion, both 1-nitropyrene and 6-nitrobenzo[a]-pyrene have been shown to transform cultured human diploid fibroblasts to a state of anchorage-independent growth and cellular invasiveness that is indicative of their potential for neoplastic growth. Since anaerobic conditions were essential

for this transformation and because the frequency could be augmented by xanthine oxidase, it appears that nitroreduction may be an essential metabolic activation pathway for these nitroarenes. The xanthine oxidase-mediated conversion of 1-nitropyrene to a DNA-binding species (18) and the decreased mutagenicity of nitroarenes in nitroreductase-deficient bacteria (8,15–17) support this conclusion and together suggest that arylamine–DNA adducts may be responsible for the biological activity of this class of compounds. This hypothesis is consistent with the recent identification of N-(deoxyguanosin-8-yl)-1-aminopyrene as the major DNA adduct formed in the xanthine oxidase/1-nitropyrene *in vitro* reaction (19), in the genome of *S. typhimurium* TA1538 incubated with 1-nitropyrene (19), and in the reaction of N-hydroxy-1-aminopyrene with DNA (Beland and Howard, unpublished observation). In addition to the mutagenic potential of nitro-PAH's in *S. typhimurium* and the transformation potential of these compounds demonstrated in the present study, nitrated pyrenes have been shown to cause mutations in cultured mouse lymphoma cells (30), Chinese hamster lung cells (31), and *Saccharomyces cerevisiae* (32). These compounds also induce sister chromatid exchanges in cultured Chinese hamster ovary cells (33), induce unscheduled DNA synthesis in HeLa cells (34), and induce local tumors in male rats (35). These observations support the hypothesis that further environmental contamination by nitrated PAH's may pose a health risk to humans.

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17

IN VITRO TRANSFORMATION OF HUMAN CELLS: MODULATION OF EARLY GENE EXPRESSION PRECEDING CARCINOGEN-INDUCED EVENTS

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I. INTRODUCTION

Many investigators have reported on the transformation of human cells *in vitro* by different types of carcinogenic insults (Milo and DiPaolo, 1978; Kakunaga, 1978, Zimmerman and Little, 1981; Namba, 1982; Maher *et al.*, 1982). Many of these investigators have treated the cells when they were proliferating

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in a logarithmic stage of growth (Silinskas *et al.*, 1981; Sutherland *et al.*, 1981; Namba, 1982). However Zimmerman and Little (1981), Borek (1980), Greiner *et al.*, (1981), and Milo and DiPaolo (1978, 1980), using either an amino acid-deficient growth medium or a small amount of fetal bovine serum-supplemented growth medium, blocked the cells in a G_1 stage of the cell cycle, released the cells from the block, and treated the cells with a carcinogen as they exhibited S-phase entry.

Data from Milo and DiPaolo (1980) suggest to us that cells released from G_1 block and treated in G_2 (4.5 h) or M (1.5 h) were not transformed by the carcinogen. Moreover, there is a period in early G_1 that the cells are refractory (will not respond to the carcinogenic insult). The movement of cells from G_1 block in the cell cycle following release from the block into S phase was determined to be 10 h in length. The intracellular localization of a cell cycle-regulatory protein, calmodulin (Yasuhanu and Hidaka, 1982; Cheung, 1982), was followed by indirect immunofluorescence, over the time of release of the cells from the G_1 block into S. For a 2-h period of time prior to release of the cells from the G_1 block and 4 h into S, the cells were treated every hour for 1 h with methylazoxymethanol acetate (MAMA). This carcinogen treatment during a 16-h time span was followed in order to discern whether a programmed release of the cells from G_1 block would alter the extent of carcinogenic response to the treatment as measured by the expression of anchorage-independent growth of carcinogen-treated cells.

II. METHODS OF PROCEDURE

A. Cell Cultures

Neonatal human foreskin fibroblast cell populations (HNF) were seeded into 25-cm^2 flasks and produced confluent monolayers in 48 h (Riegner *et al.*, 1976). The cells were serially passaged and the incremental radiolabeling index determined as a measure of the growth phase (Cristofalo and Sharf, 1973). The cultures were maintained in complete growth medium composed of Eagle's minimum essential medium (MEM) supplemented with nonessential amino acids, sodium pyruvate, gentocin, glutamine, 25 m*M* Hepes at pH 7.2 (Milo and DiPaolo, 1980), and 10% fetal bovine serum. The routine serial passaging of the cells at 1:4 split ratios was done in the aforementioned complete growth medium (CM).

B. Preparation of Cells for G_1 Block

To arrest the cell population in the late G_1 phase of the cell cycle 10 h prior to S phase, the logarithmically growing cell population at 70% confluent density was transferred by seeding the cells into a modified Dulbecco's minimum essential medium (DM) deficient in arginine and glutamine for 24 h. Dialyzed fetal bovine serum (d-FBS) was prepared by dialysis against DM deficient in arginine and glutamine. DM Was supplemented with 10% d-FBS over the 24-h period in G_1. Sister cultures were prepared for each time point, and one culture was treated with [*methyl*-^{3}H]thymidine (60 Ci/mmol) at 1.0 μCi/ml. Samples were taken every hour from the time the cultures went into the block until midway into S period 38 h later. The other sister culture was treated with the carcinogen in the G_1 block, at time of release from the block, and midway into S period. All of the cultures were incubated in a 4% carbon dioxide-enriched air atmosphere at 37°C.

C. Chemical Preparation

The chemicals of interest--insulin (IN at 0.5 U/ml) methylazoxymethanol acetate (MAMA), 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), and benzamide (BZ)--were solvated in either ethanol or acetone (Milo *et al.*, 1981) and used immediately. MAMA (3.6 μg/ml), MNNG (0.5 μg/ml), or BPDE I (0.114 μ*M*) were added to the cultures at the time the cells were released from the block. In addition, MNNG (Milo and DiPaolo, 1980) and BPDE I (Tejwani *et al.*, 1982) were added when the cells were in S.

Finally, MAMA was added at each hour for 1 h of treatment from 2 h prior to the release point until the cells were 4 h into S. Benzamide was added at 1 m*M* final concentration at the G_1 cell block period after release from the block, removed at S-phase entry, and then added again in a sister culture at S-phase entry.

D. Transformation Regimen

Proliferating cell populations at a cell density of 5000 cells/cm^2 were transferred from CM to DM minus arginine and glutamine and blocked in G_1 (Milo and DiPaolo, 1980; Zimmerman and Little, 1981). The cells were released from the block by removal of DM medium and readdition of CM (plus deficient amino acids) with and without IN, and 10 h later the cells exhibited S-phase entry. Treatment with the carcinogen was carried out as described previously. The treated populations were serially passaged (Milo and DiPaolo, 1978; Zimmerman and Little, 1980; Silinskas *et al.*, 1981) into CM containing 8x nonessential amino acids and 2x vitamins for 20 PDL, and at this time the treated cells were seeded into a semisolid medium to measure anchorage-independent growth (0.33% agar).

E. Anchorage-Independent Growth

The treated populations were serially passaged into 2 ml of soft agar (0.33%) containing Dulbecco's modified lo-Cal medium (Biolabs, Northbrook, Illinois) supplemented with 20% FBS (Milo *et al.*, 1981) over a 5-ml 2% agar base containing McCoy's 5A supplemented as described previously (Milo *et al.*, 1981) with 20% FBS.

The treated cultures were kept at a high humidity in a 4% carbon dioxide-enriched atmosphere at 37°C. After 21 days, colonies that contained ≥50 cells/colony were scored as positive.

F. Assay for Neoplasia

After counting the colonies in soft agar they were removed, pooled, dispersed into single-cell suspensions, and seeded into 25-cm^2 flasks containing 2 ml of CM supplemented with growth additive and 10% FBS. These transformed populations were evaluated for their neoplastic potential in *nude* mice (Donahoe *et al.*, 1982). The *nude* mice were irradiated with 450 rads (from a ^{137}Cs source) prior to the injection of the transformed cells. After 6 weeks, tumors were counted and excised for histopathological evaluation.

G. Calmodulin Localization

Cells seeded at ∿10,000 cells/cm^2 were fixed in 3% phosphate buffer formalin at pH 7.2 for 30 min. The slides were then postfixed in methanol at 20°C for 10 min, rinsed with PBS, and indubated 1 h at 37°C with the primary antibody toward calmodulin. This antibody was prepared against rat testis calmodulin protein in sheep. After rinsing the fixed cultures extensively with PBS, the secondary reagent, FITC-conjugated rabbit anti-

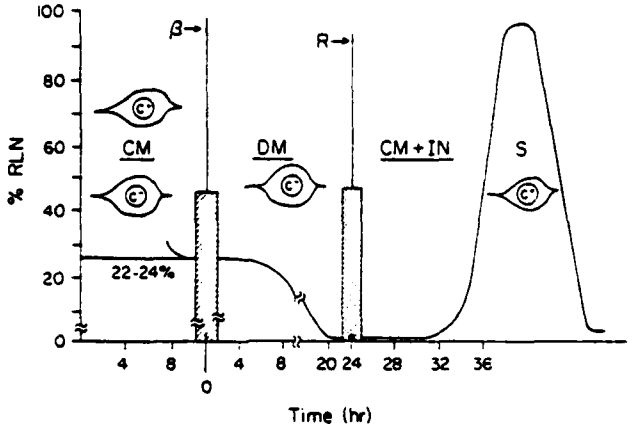

Fig. 1. Graphic representation of behavior of cells leaving the block (R) G_1 traversing to S. β represents point when proliferating cells in log growth are fed with DM minus arginine, glutamine, and d-FBS. R is the point in time (h) when the cells are released from the G_1 block and the incremental part of the cell population proceeds toward S phase of the cell cycle. This population contains the maximum number of cells that exhibit positively fluorescing antisera against calmodulin localized in the nucleus at this time point. From R to the time the population exhibits S-phase entry ([*methyl*-^{3}H]thymidine radiolabel nuclei), there is no further increase in nuclei that fluoresce positive for the presence of calmodulin. S represents the point when the cell population enters S phase of the cell cycle. The period R to S will be designated as S-phase entry time, and β to R will be designated as G^1 transient period or late G_1 period.

sheep IgG, was incubated with the cells for 1 h at 37°C. Again the slides were washed with PBS and examined under a Zeiss epifluorescent microscope with 485-nm exciter filters and 520- to 560-nm barrier filters (Dedman *et al.*, 1978; Markle *et al.*, 1981).

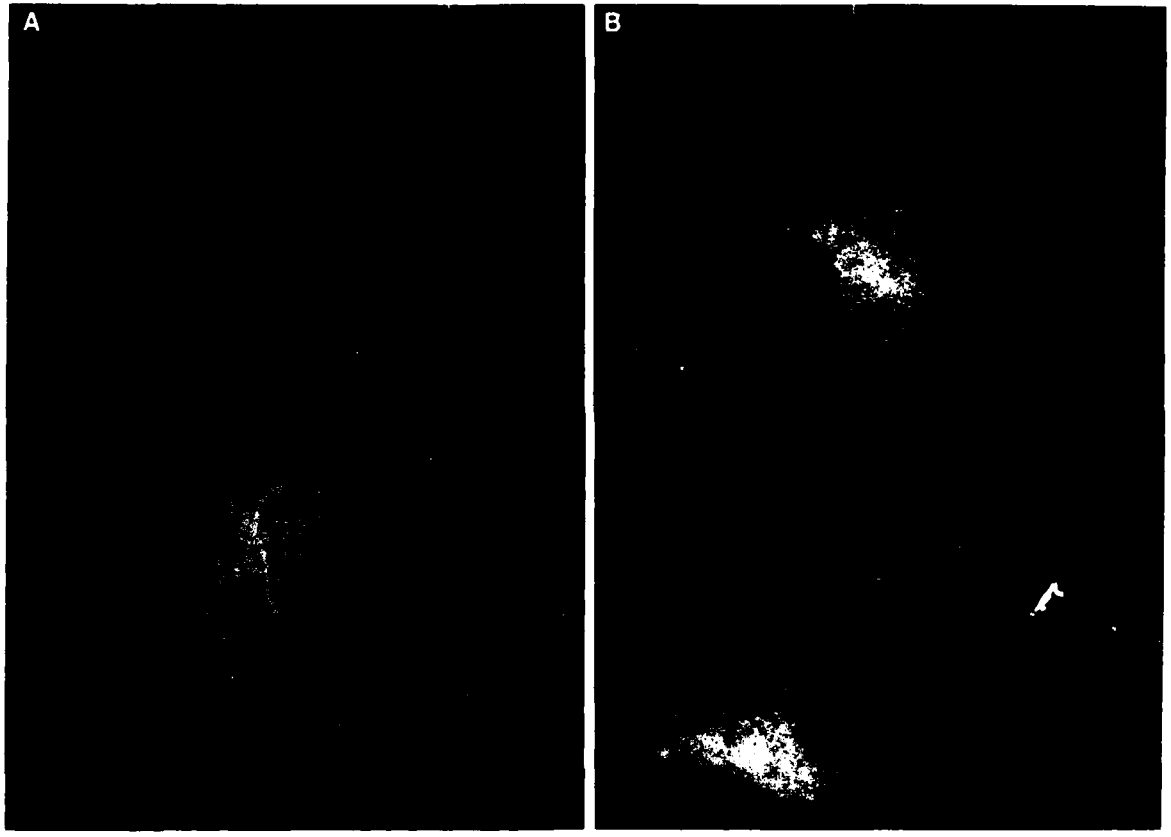
A
B

Fig. 2. (A) Photomicrograph of a fibroblast exhibiting fluorescence localized in the cytoplasm of the cell but not in the nuclear area (for the presence of calmodulin). The fluorescence is indicative of the localization of calmodulin in these cells when they are at point R (Figs. 1 and 3). (B) Localization of the fluorescence (calmodulin) in the nuclear area, and less intense fluorescence in the cytoplasmic area. These cells were evaluated 4 h after release from the block (DM, minus arginine and glutamine) (×35).

III. RESULTS

Proliferating populations radiolabeled with [*methyl*-^{3}H]-thymidine exhibited an incremental radiolabeling index from 22 to 24% (Fig. 1). When the CM medium over these populations was replaced with DM (point β, Fig. 1), the radiolabeling index (Cristofalo and Sharf, 1973) dropped to ≤0.1%. At point R (Fig. 1), the DM medium was replaced with CM (Milo *et al.*, 1981), and IN was added to the CM. After 10 h the untreated and treated cells exhibited S-phase entry (Milo and DiPaolo, 1980). Calmodulin, a cell-regulatory protein (Cheung, 1982) associated with cell proliferation, localizes in the nucleus before the cells enter S. The protein can be located intracellularly by indirect immunofluorescence, and following release of the cells from the block 2 h later fluorescence staining properties for calmodulin were observed in the cytoplasm of the cells (Fig. 2A). Four hours later, the nuclei fluoresced positive for the presence of the protein (Fig. 2B). Six hours after release from the block the maximum number of positive fluorescing calmodulin nuclei were observed. No further increases in the number of positive fluorescing calmodulin nuclei (Table I) were found after the 6-h lapse of time. We followed these events to S-phase entry, ($G_1^{10\text{-}11\ h}$). Concomitantly we examined the radiolabeling index over this same time period. At $G_1^{0\text{-}1}$ the radiolabeling index was ≤0.1%. At $G_1^{10\text{-}11}$ the incremental radiolabeling index increased from ≤0.1 to 3%, and at $S^{13\text{-}14}$ the 1-hr Δ radiolabeling index was 28% for IN-treated cells.

Treatment of the sister cell populations with the carcinogens MNNG, BPDE I, and MAMA was initiated at $G_1^{0\text{-}1}$ and $S^{12\text{-}13}$ following release of the cells from the block (Point R, Fig. 3). Transformation was measured after 20 population doublings by the ability of the treated cells to grow in soft agar (as

TABLE I. The number of Calmodulin-Positive Fluorescing Nuclei Followed as a Function of Time of Release from the Block

% Calmodulin Fluorescing Nuclei[a]		
-IN[c]	+IN[c]	Time in G_1 $t-t_1$[b]
0	0	G_1^{0-1}
1	2	G_1^{3-4}
25	50	G_1^{6}
30	52	G_1^{8-9}
31	51	G_1^{9-10}
33	50	G_1^{10-11}

[a] *The number of calmodulin-positive fluorescing nuclei/number of nonfluorescing nuclei × 100 expressed as a percentage.*

[b] *The notation G_1^{0-1} to G_1^{10-11} refers to the G_1 part of the cell cycle, and $t-t_1$ was used to describe the beginning and end of the sampling time in hours following release from the block at point R (Fig. 1).*

[c] *-IN, Cultures released from the block receiving no insulin treatment; +IN, sister cultures receiving insulin treatment. These values are mean values for three readings.*

described herein). The number of colonies observed from G_1^{0-1}-treated populations treated with MNNG, BPDE I, and MAMA was zero (undetectable). At point G_1^{6-7}, when the optimum number of calmodulin-positive nuclei were observed (Table I), the incidence of anchorage-independent growth of these carcinogen-treated cells was 18 ± 3.0 for MAMA-treated cells, 0.0 for MNNG-treated cells (undetected), and 0.0 for BPDE-I-treated cells (undetected). When the populations were treated with the carcinogen at S-phase entry (G_1 + S^{10-11}) with either BPDE I or MNNG, the colony-forming incidance of these treated cells in soft agar was 26 colonies/10^5 BPDE I-treated cells (Tejwani et

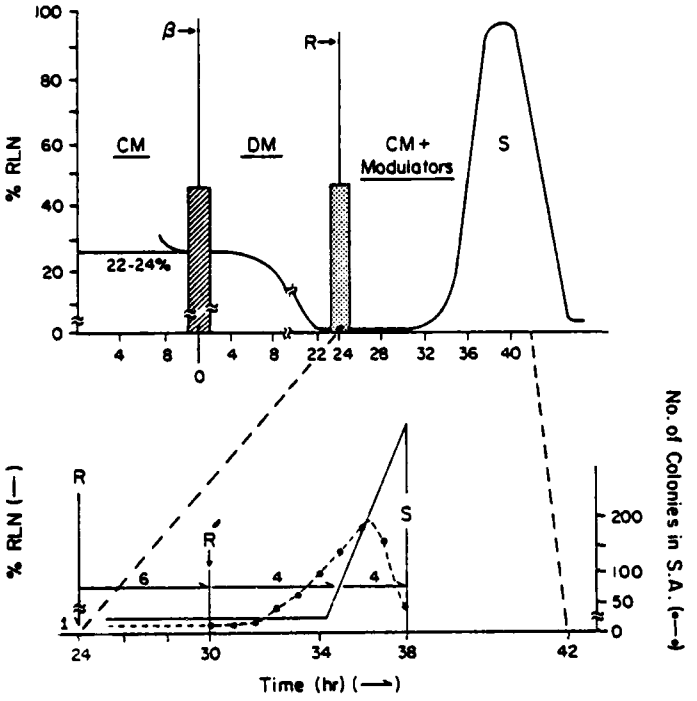

Fig. 3. The percentage of radiolabeled nuclei (% RLN) was plotted as a function of time following release from the cell cycle block at 24 h. The sections were divided into 6-h and 4-h periods. Six hours after release from the block at R' the maximum number of calmodulin-positive fluorescing nuclei was determined. The carcinogen treatment period was for 1 h in length from R' to S, 6-18 h after R (4 h into S). The number of colonies per 50,000 cells seeded into soft agar (S.A.) was plotted as a function of the length of treatment (1 h) at various time points, 30-38 h. These are mean values for three wells (see footnotes to Table II). The data presented here represent a combination of data from Table II and Fig. 1.

al., 1982) and 2.0 colonies/10^5 MNNG-treated cells (Milo *et al.*, 1981). When MAMA was used to treat populations at S-phase entry for 12 h we observed 700 colonies/10^5 cells (Kuhn *et al.*, 1982).

We therefore decided to titrate sister populations (Table II) with MAMA from G_1^{0-1} through to S^{13-14}. We treated the

TABLE II. Comparison of Treatment Time Following Release from G_1 Block (Hour R) into S Phase of the Cell Cycle with Number of Colonies that Exhibit Anchorage-Independent Growth

Number of colonies expressing anchorage-independent growth[a]		
-IN[b]	+IN[c]	Time $G_1{}^{t-t_1} + S^{t-t_1}$[d]
0	0	$G_1{}^{0-1}$
0	0	$G_1{}^{3-4}$
1.4 ± 1.5	18 ± 3.0	$G_1{}^{6-7}$
1.4 ± 1.5	15 ± 2.3	$G_1{}^{7-8}$
1.4 ± 1.5	30 ± 17.0	$G_1{}^{8-9}$
20.3 ± 9.2	45 ± 3.1	$G_1{}^{9-10}$
12.3 ± 0.6	36 ± 2.5	$G_1 + S^{10-11}$
10.3 ± 3.5	150 ± 27.0	S^{11-12}
9.5 ± 3.0	271 ± 15.7	S^{12-13}
8.0 ± 0.0	65 ± 4.0	S^{13-14}

[a] *Treated cells (50,000) at 20 PDL were seeded into 2 ml of 0.33% agar overlayer over 5 ml of 2.0%. The growth medium in the agar overlayer was Dulbecco's lo-Cal medium (Milo et al., 1981) over McCoy's 5A in the 2.0% base; each contained supplements and FBS as described in Section II. Untreated cells seeded in the same manner yielded no colonies from 10^5 cells.*

[b] *The cells were released from the G_1 block, and no IN was added prior to administration of the carcinogen MAMA.*

[c] *The cells were released from the block and IN added prior to the administration of the carcinogen. The values reported here are mean values for three wells ± 1 standard deviation.*

[d] *The designation $G_1{}^{t-t_1} + S^{t-t_1}$ was used to describe the phase of the cell cycle such as G_1 or S, and the superscript $t-t_1$ is the beginning and end of the treatment time with carcinogen during that phase of the cell cycle. For example, $G_1{}^{0-1}$ is the time in G_1 when the cells were released from the block and were treated for 1 h. At this time the 1-h Δ radiolabeling index, using [methyl-^{3}H]thymidine (Milo and DiPaolo, 1980), was <0.01%. At the time of treatment, S^{13-14}, the incremental radiolabeling index was 22% for -IN carcinogen-treated cultures and 28% for +IN treated cultures.*

cells from the time of release, G_1^{0-1} for 1 h duration at each hour up to S^{13-14}, that is, 13-14 h after release from G_1 (point R, Fig. 1 or Fig. 3). Over this time period we found that -IN + MAMA-treated cells did not exhibit an increase in colony formation in soft agar for G_1^{6-7}-treated populations over the G_1^{0-1} period; that is, no colonies were found in soft agar. Later, at S^{12-13} time period there was an increase in colony formation to 9.5 ± 3.0 colonies per 50,000 seeded cells. Insulin pretreatment of cells to be treated with MAMA at time point S^{12-13} resulted in the formation of 271 ± 15.7 colonies per 50,000 seeded cells (Table II). The 1-hr Δ radiolabeling index at this time point was 28% (see footnotes to Table II). At S^{13-14} treatment period there was a substantial decrease from 271 ± 15.7 colonies to 65 ± 4.0 colonies. However, the 1-h incremental radiolabeling index increased to 75%. We did not at this time carry the experiments beyond this point.

Previous experiments (Milo *et al.*, 1981), with treatments in the later part of S with MAMA, MNNG, or BPDE I, prove that the treated cells in this time period were less responsive to the carcinogenic insult; that is, the treated cells did not exhibit anchorage-independent growth. The relationship between the release of the cells from the blocks and treatment regimen with MAMA presented in Fig. 3 illustrates the profile of colony formation versus treatment time (Table II) over a 14-h treatment period of time. Table II is a graphic representation of the treatment regimen from point R through to midway into S. Furthermore, data not presented here have revealed that the carcinogenic response can be interfered with following the carcinogen treatment with BZ. Benzamide at 1 m*M*, added to the cells at point R and removed at S (Fig. 3), reduced the number of colonies formed to 67% of non-BZ-treated MAMA-treated cells. When BZ was added at point $G_1 + S^{10-11}$ and removed at S^{14} (i.e., 4 h later into S), it reduced the MAMA-induced colony-forming

frequency in soft agar from 10^3 colonies/10^5 seeded cells to <2 colonies/10^5 cells (Kun and Milo, 1982). The 1-h Δ radiolabeling index of BZ carcinogen-treated cells over the treatment time was not altered when compared to the sister MAMA-treated cultures containing no BZ. There was no apparent cytotoxicity exhibited by the 1 m*M* BZ-treated cultures or change in finite population doublings of these BZ-treated cells (35 ± 5 population doublings). BZ-treated cell populations would not grow in soft agar. Injection of the MAMA-transformed cells into a *nude* mouse yielded a tumor that was excised from the mouse and evaluated as an undifferentiated mesenchymal tumor. The frequency of tumor formation was two tumors per 16 mice that receive the injection (Donahoe *et al.*, 1982).

IV. DISCUSSION

The transformation of human foreskin fibroblasts by carcinogen insults appears to be an operational phenomenon that is logistically reproducible. There are several reports in the literature that randomly proliferating cell populations can be transformed following carcinogen administration (Kakunaga, 1978, Sutherland, 1981, Maher *et al.*, 1982; Namba, 1982). There are reports also by other investigators that blocking human diploid cell populations in G_1 by specific amino acid deprivation (Milo and DiPaolo, 1978, 1980; Zimmerman and Little, 1981; Greiner *et al.*, 1981) or by reducing the fetal bovine serum requirement (Borek, 1980) followed by the addition of modulators (compounds that modify intracellular biochemical events without damage to the DNA), enhances the carcinogenic response. The heightened response to the carcinogen treatment of the cells following treatment with the modulators such as IN during the late G_1 phase of the cell cycle and into

S permits us to probe the system with the carcinogen to identify the time of optimum response to the carcinogen. Maher *et al.* (1982) have found, using 6-thioguanine mutagenic selection procedures, that the slope changes immediately prior to S-phase entry, and there appears to be a correlation between the number of mutants observed at this time and the number of transformants (express anchorage-independent growth).

We (Tejwani *et al.*, 1982) completed carcinogen-DNA adduct studies on cell populations that will either respond to the carcinogenic insult and will exhibit anchorage-independent growth (responsive) or will not exhibit anchorage-independent growth (refractory). We found, in responsive cells treated with BPDE I in early S, the period of heightened responsiveness to carcinogen insult, that the carcinogen-dG adduct formation compared to refractory treated populations was quite similar. Furthermore, the persistence of adducts in the refractory or responsive carcinogen-treated cells over a 24-h period did not appear to be a logical mechanism to explain why we observe the differences in formation of colonies in soft agar, for BP-DNA binding did not change in either treated cell population over this time period. Moreover, Zimmerman and Little (1981) suggest that specific amino acid deprivation may contribute to heighten response to the carcinogen treatment. This may indeed occur; however, responsive or refractory cells, both those blocked in G_1 by amino acid deprivation and those released from the block in G_1, show no statistical significant differences in binding of the radiolabel carcinogen to the DNA.

Maher *et al.* (1982) have shown that there is a dose-dependent mutagenic response to UV light (254 nm) when cells were treated in late G_1 3 h prior to onset of S and these treated cell populations were examined for their capability to grow in soft agar. It is interesting to note that the cells we have treated with MAMA at 10 h prior to onset of DNA synthesis

exhibit anchorage-independent growth, whereas MNNG and BPDE I treated cells do not exhibit this characteristic. In all three treatments with BPDE I, MNNG, and MAMA, the time of optimum responsiveness to the carcinogenic insult appears to be 2-3 h into S. This response appears to be wiped out by BZ treatment, lowering the expression of anchorage-independent growth from ~700 colonies/10^5 treated cells seeded in soft agar to <2 colonies/10^5 cells. Furthermore, we (Kuhn *et al.*, 1982) have found that BZ ameloriates the poly(ADP)ribosylase-phosphorylated modification of acidic nuclear proteins of responsive cells.

We suspect that a complex series of events occurs in a programmed manner as a function of time from the time of adding a modulator through to the expression of the early carcinogenic events. If, for example, one of these events is modified by the administration of BZ while DNA damage, persistence of adducts, and repair of damage is in process, the cell responds to the insult as a reversible or irreversible toxic event. We propose that modification of nuclear protein covering the carcinogenic site sets in motion events leading to the expression of a carcinogenic response (precancerous state), followed by an expression of anchorage-independent growth and neoplastic potential. This of course presupposes that the correct genetic damage (carcinogen-DNA adduct) was formed, and "error-prone repair" is functional in S followed by chromosomal transposition (Klein, 1981). In any event, calmodulin, a cell-regulatory protein in this system, apparently is active in the nucleus, is expressed early in late G_1 prior to the onset of scheduled DNA synthesis, and prefaces any subsequent event leading to cellular proliferation and fixation of the genetic damage in expression of a carcinogenic event.

V. SUMMARY

Human foreskin fibroblast populations blocked in G_1, released, and treated with methylazoxymethanol acetate (MAMA) from the time of release (late G_1) for 1-h treatment intervals until 4 h into S, exhibited a differential sensitivity to MAMA treatment at the different treatment times. A heightened response to the carcinogen treatment was not detected until calmodulin, a cell-regulatory protein, was optimally present in the nuclei of the late G_1-treated cells 6 h after release from the G_1 block. Moreover, there was a distinct increase in the number of transformed phenotypes (cells that will grow in soft agar), observed when the cells were treated with MAMA at the onset of scheduled DNA synthesis. The time at which these treated cells were optimally responsive to a carcinogenic insult was 12-13 h after release from the block 2-3 h into S. Interestingly, this was followed by a decrease in the expression of anchorage-independent growth when the cells were treated 13-14 h after release from the block 4 h into S. Benzamide interfered in the process when added at the onset of S, and the resultant carcinogen-treated population did not exhibit a comparable increase in expression of anchorage-independent growth. Cells treated with MAMA at the point of release from the block G_1^{0-1} to G_1^{3-4} did not express anchorage-independent growth.

It is proposed that the heightened presence of calmodulin in the nuclei 4 h prior to the onset of scheduled DNA synthesis is a cell-regulatory function that sets in motion a complex series of events (program) in carcinogen-initiated human fibroblasts that leads to a subsequent carcinogenic response.

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Cell Biology

Cell cycle-dependent intervention by benzamide of carcinogen-induced neoplastic transformation and *in vitro* poly(ADP-ribosyl)ation of nuclear proteins in human fibroblasts

(cell synchronization/S phase/anchorage-independent growth)

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ABSTRACT Human fibroblasts were subjected to nutritionally induced G_1 block, followed by release and subsequent entry into S phase, and exposed to nontoxic concentrations of carcinogens in early S phase. Cell transformation occurred as determined by early morphologic cell alterations, anchorage-independent colony formation, cell invasiveness, and augmentation of Ab 376 human malignancy-specific cell-surface antigenic determinant. Methylazoxymethanol acetate was the most potent transforming agent at doses that were negative in toxicity tests. Benzamide (10 μM intracellular concentration), a specific inhibitor of poly(ADP-ribose) polymerase, prevented transformation in a cell cycle-specific manner, maximal prevention coinciding with early S phase, also characteristic of maximal susceptibility to transformation. Neither an interference of carcinogen deoxyguanosine nucleoside adduct formation nor a chemical reaction between benzamide and carcinogens was detected. Methylazoxymethanol acetate at transforming but nontoxic dose partially inhibited poly(ADP-ribosylation to about the same extent as benzamide. However, simultaneous exposure of cells to both agents in early S phase, resulting in the prevention of transformation, augmented poly(ADP-ribosyl)ation above the controls. Enzymatic activities ran parallel with the formation of DNA-associating polymer-nonhistone protein adducts that are assumed to regulate the physiological function of chromatin at the structural level.

A significant increase in poly(ADP-ribosyl)ation of nonhistone proteins was found in an early precancerous state of hamster liver (1) and in regenerating rat liver (2), suggesting that cellular regeneration may be a common reason for the accelerated enzymatic rates. Cellular regeneration is known to augment the number of cells in S phase and this has been correlated with the facilitation of neoplastic transformation in organs (3) and in cultured C3H/$10^1/_3$ CL8 mouse fibroblasts (4). Poly(ADP-ribosyl)ation also exhibits a cell cycle-dependent oscillation, a maximum coinciding with S phase after a release of G_1 block (5). These observations tend to suggest an as yet undefined connection between (poly ADP-ribosyl)ation, cell cycle, and neoplastic transformation.

Ontogenic development (6), the effect of developmental hormones (7–9), and differentiation (10) also coincide with changes in rates of poly(ADP-ribosyl)ation of mainly nonhistone proteins (11, 12) that are thought to regulate selective gene expression (13).

Identification of poly(ADP-ribose) as a unique nucleic acid (14, 15) that is covalently bound to presumably DNA-associated proteins may provide a molecular model of chromatin regulation. Ionic environment-dependent helical polymer chains of poly(ADP-ribose) (15) may act as crosslinking agents between regulatory proteins and, depending on their nature and localization, could alter chromatin conformations, expressed as karyological changes that accompany the cell cycle in normal and malignant cells (16, 17). The poly(ADP-ribosyl)ation-dependent variation in nucleosomal structures (18) tends to support the proposed regulatory mechanism.

We examined the possible participation of poly(ADP-ribosyl)ation of chromatin proteins in carcinogen-induced oncogenic transformation of synchronized human fibroblasts (19–21). This model was chosen because we intended to obtain information that could be relevant to human neoplasia. If poly(ADP-ribosyl)ation of certain chromatin proteins in the S phase plays a role in the regulation of carcinogen-induced oncogenic transformation, then a selective inhibitor of poly(ADP-ribose) polymerase, benzamide (22), would be expected to alter transformation. A preliminary report has appeared (23).

MATERIALS AND METHODS

Cells and Culture Conditions for G_1 Block/Release. Primary human fibroblasts were prepared (24) and cultured as reported (25). These cells have a finite replicative capacity of 35 ± 7 population doublings, which is 22.4 hr, plating efficiency between 95 and 100%, and relative colony (defined as 50 cells)-forming efficiency of 20–21%. For each series of experiments the average yield of cells prior to the soft-agar growth test was $3 \times 10^6 \pm 20\%$ per flask (73 cm^2 each) and for biochemical studies the number of cells was scaled up to about 20×10^6. G_1 block (26) was induced as described (19). It is critical that the number of population doublings at the initiation of G_1 block must not exceed 5 and the doubling time 23 hr; otherwise resistance to transformation by carcinogens may develop (24) and the variations in poly(ADP-ribosyl)ation will differ from results reported here. In G_1 block (19) radiolabeling fell to 0.1% of controls within 24 hr. The G_1 block was released by refeeding with the minimal essential medium, which also contained 0.5 unit of insulin per ml (see Fig. 4). After two washings with minimal essential growth medium, from which bovine serum was deleted, cultures were divided (1:2) and culturing commenced after the addition of 2× concentrated essential vitamins, 9× concentrated essential amino acids, 0.2% $NaHCO_3$, 50 μg of gentocin per ml, and 20% fetal serum until confluence was approached and serial passages were continued (1:10) in the enriched minimal essential medium (see Fig. 4). Seeding (4×10^5 cells per plate) into soft agar (19) was carried out after 16–20 population doublings.

Exposure to Carcinogens and Benzamide. Exposure to carcinogens and benzamide was done 10 hr after the release of the

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Abbreviation: RCF, relative cloning frequency.

metabolically induced G_1/S block (Fig. 4) and exposure lasted 10 hr, followed by three washings and refeeding with fresh media (25).

Cellular Toxicity. Cellular toxicity, tested at least at six concentrations of drugs, was determined by effects on cloning frequency (26). Relative cloning frequency (RCF_1) was defined as the ratio between cloning efficiency of controls and cultures exposed to drugs, and RCF_{50} was defined as the concentration of drugs that caused a 50% decrease of cloning frequency. Toxicity was the same in random and synchronized cultures. The time of exposure to drugs was 24 hr, even though in the transformation experiments (Fig. 4) this was only 10 hr, providing an extra margin of safety for the determination of nontoxic doses, which were further tested by the absence of effects on plating efficiency and doubling time.

Criteria for Phenotypic Transformation. In addition to colony counts on soft agar (19, 25), criteria for phenotypic transformation were cellular invasiveness (27), determined in six parallel tests per experiment, and the immunofluorescence test for human malignancy-specific monoclonal cell-surface antigenic determinant Ab 376 (28, 29) on cells obtained from soft agar clones (unpublished data). The tumor take in *nude* mice (19) was identical with the incidence of tumor formation by surgically obtained human fibrosarcomas, cultured parallel with transformed fibroblasts, and the low incidence (20–30%) is probably explained by genetic differences between the human cells and the host (30).

Biochemical Procedures. Biochemical procedures related to poly(ADP-ribose) were the same as published (1, 2, 6, 11, 12, 14, 15, 31–33). Intracellular benzamide (^{14}C-labeled) was determined after removal of the adsorbed drug, lysis in NCS tissue solubilizer, methanol extraction, and reversed-phase chromatography on a C_{18} (Bondpak) column with a 0–60% methanol/H_2O linear gradient. Fibroblasts were permeabilized by lysophosphatidylcholine (34), which did not interfere with enzyme assays.

[^{14}C]Benzamide (1C) (4.29 μCi/mmol, 5 μCi/ml of solution; 1 Ci = 3.7×10^{10} Bq) was purchased from Pathfinder Labs, St. Louis, MO. [1,4-^{14}C]Methylazoxymethanol acetate (specific activity, 115 mCi/mmol) was kindly provided by F. Cazer (Ohio State University). The monoclonal antibody to Ab 376 was a generous gift of S. Ferrone and the secondary reagent (fluoroisothiocyanate-conjugated goat anti-mouse IgG) was purchased from Miles.

RESULTS

Prevention of Transformation. Transforming (RCF_1) and toxic (35) (RCF_{50}) doses of methylazoxymethanol acetate were 7 and 27 μM, respectively, and for benzamide the doses were 1 mM (added externally to cell cultures) and 4.75 mM, respectively. These values for the carcinogen or benzamide did not change if the two agents were combined, as under conditions that prevented transformation. Identical toxicity analyses were performed with five additional carcinogens (Table 1). Benzamide (added externally at 1 mM, corresponding to 10 μM intracellular concentration) counteracted transformation. Benzoate at 1 mM had no influence on the transformation nor did it have an effect of its own on fibroblasts (not shown).

Evidence of Cellular Transformation. The characteristic morphology of fibroblasts (Fig. 1, group 1) was markedly altered when cell cultures were exposed to RFC_1 doses of carcinogens after five cell doublings. Benzamide in cell cultures inhibited this phenotypic change (Fig. 1, group 3), whereas the cell morphology shown in Fig. 1, group 1, remained unaltered by benzamide alone. Cells shown in Fig. 1, group 3, have become resistant to transformation by reexposure to another cycle

Table 1. Inhibition by benzamide of carcinogen-induced cell transformation as determined by colony counts on soft agar

Carcinogen	Experiments, no.	Experimental conditions	Transformed cell colonies, no. per 10^6 cells
1	5	Methylazoxymethanol acetate (7.0 μM)	758 (±58)
	5	+ Benzamide	1 (±0.3)
2	2	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (0.7 μM)	39 (±6)
	2	+ Benzamide	0 (±0.2)
3	2	7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzopyrene (0.33 μM)	26 (±2)
	2	+ Benzamide	0
4	2	β-Propiolactone (28 μM)	28 (±3)
		+ Benzamide	0
5	1	1,1-Dimethylhydrazine (167 μM)	103 (±9)
	1	+ Benzamide	1 (±0.2)
6	2	3-Hydroxy-1-propanesulfonic acid γ-sultone (122 μM)	43 (±5.5)
	2	+ Benzamide	1.0 (±0.3)
7	5	No additions	0
8	5	Benzamide	0

Both carcinogens and benzamide were present in RCF_1 concentrations.

and G_1 block/release and carcinogen (in S phase) in the absence of a new dose of benzamide, and this resistance is likely to be due to a modified genetic trait because residual benzamide was not present after three or four cell doublings. Cellular invasiveness (27) of transformed colonies grown in soft agar is shown in Fig. 2, and the fluorescence-antibody binding test (29) performed with the Ab 376 monoclonal antibody (28) is shown in Fig. 3*B*.

Time Course of Exposure of Fibroblasts to RCF_1 Doses of Benzamide and Methylazoxymethanol Acetate. The time course of exposure of fibroblasts to RCF_1 doses of benzamide and methylazoxymethanol acetate as related to G_1/S block and its release is illustrated in Fig. 4. The partial antagonism by benzamide, added during G_1 block and in early S phase (lines A and B) is most probably explained by the partial retention of benzamide in cells even after several washings (see *Materials and Methods*).

DNA Adduct Formation. DNA adduct formation (36–38) was studied with two labeled carcinogens (carcinogens 1 and 3 in Table 1) under identical conditions required for transformation. The quantities of adducts in experiment 1 were 90.6 and 96.0 pmol/mg of DNA (22×10^3 and 21×10^3 cpm of ^{14}C) and in experiment 2 were 45 and 41 pmol of methylazoxymethanol acetate per mg of DNA (11.5×10^3 and 11×10^3 cpm). Rates of transformation were similar in both cases. In each experiment the second value was obtained in the presence of benzamide. One of the labeled products of methylazoxymethanol acetate was [^{14}C]methyl-*O*^{6}-guanosine, 5–9 adducts per 10^6 bases (cf. ref. 39). Methylation of DNA with [*methyl*-^{14}C]methionine as a methyl donor was not influenced by benzamide (results not shown). Adduct formation of DNA with [^{3}H(U)]7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzopyrene (carcinogen 3 in Table 1) yielded mainly 7β-benzopyrene diol epoxide I-deoxyguanosine (4–8 adducts per 10^6 bases) identified by chromatography (33). The quantities of these major and also of

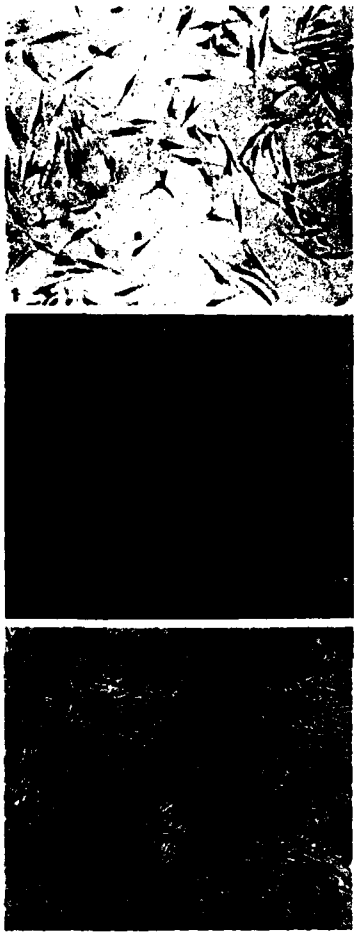

FIG. 1. Cellular morphology of normal (group 1), methylazoxymethanol acetate-treated (group 2), and benzamide- and methylazoxymethanol acetate-treated cells (group 3) as seen under phase optics. (×175.) Treatment with benzamide alone resulted in cells that were indistinguishable from group 1; therefore, they are not shown. The same morphologic effects were seen between 5 and 30 population doublings.

minor products were uninfluenced by benzamide, supporting previous results (33). Thus, the same quantities and types of carcinogen adducts occur in resistant and transformable cell cultures. Benzamide and carcinogens and their degradation products were reisolated by high-performance liquid chromatography (33), excluding chemical artefacts. Autoradiography indicated a tight association of benzamide with nuclear membrane structures.

Alkaline Sedimentation Profile of DNA. At 1.97 μM methylazoxymethanol acetate (colony formation in soft agar, 50–70 colonies per 10^5 cells) the alkaline sedimentation profile of DNA was the same in controls and in methylazoxymethanol acetate- or benzamide-treated cells when drugs were added separately or in combination (5.26×10^6 daltons). At RCF_1 (7 μM) methylazoxymethanol acetate induced an alkali instability in DNA size, as evident from the appearance of a second DNA molecular species of 1.52×10^6 daltons. However, prevention of transformation by benzamide did not coincide with reassociation to the larger size DNA; thus, no connection between apparent fragmentation and reassociation could be ascertained that correlated with transformation and its prevention.

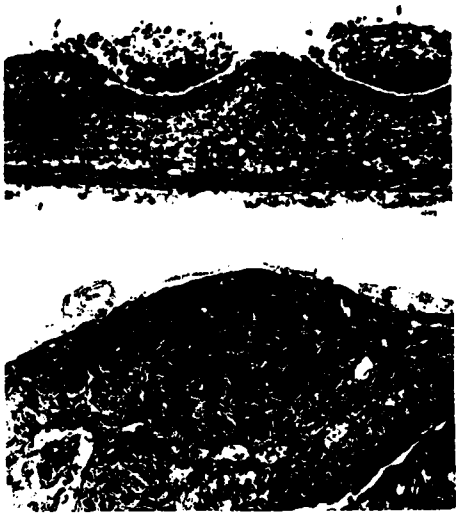

FIG. 2. Tissue invasiveness (cf. ref. 27) of methylazoxymethanol acetate-transformed fibroblasts, grown in soft agar for 20 days. (*Upper*) Normal fibroblasts placed on chicken embryo skin for 72 hr. (*Lower*) Invasive behavior after the same length of exposure to transformed cells.

Variations of Poly(ADP-ribose) Polymerase Activity and Determination of Products. After 10 hr of exposure to 1 mM benzamide in cell cultures, inhibition of enzymatic activity was similar to that observed in permeabilized cells at 10 μM benzamide. As shown in Fig. 5 methylazoxymethanol acetate at 7

FIG. 3. Detection of common antigenic determinant of human malignancies in transformed human fibroblasts by fluorescence microscopy. (*A*) Normal; (*B*) transformed (see legend to Fig. 2). Photographs were taken with a Zeiss fluorescence microscope (excitation = 485 nm, read at 520–560 nm). (×88.)

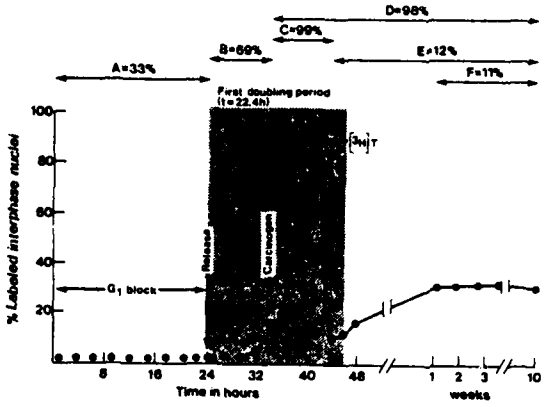

FIG. 4. G_1 block and release was followed in eight parallel cultures per experiment (4×10^6 cells per group). The times of release from G_1 block and addition of carcinogens are indicated by vertical arrows and the first doubling time is indicated by the shaded area. The exposure to benzamide is shown by horizontal arrows (top) together with the % protective effect against transformation, determined by colony counts in soft agar. Zero percent was defined when the carcinogen (methylazoxymethanol acetate) was present only. ●, [^{3}H]Thymidine ([^{3}H]T) labeling; abscissa, time (first in hours, then in weeks).

μM (Table 1, carcinogen 1) inhibited poly(ADP-ribose) polymerase activity in S phase nearly to the same extent as 10 μM benzamide, but combined exposure of cells in the S phase (see Fig. 4) to both the carcinogen and benzamide—resulting in the inhibition of transformation (Table 1)—not only restored rates to the level of controls in S phase but also augmented them. Biochemical studies (Table 2 and Fig. 5) were confined to these experimental conditions. As shown in Table 2, after incubation of permeabilized cells for 20 or 40 min, protein adducts of short chains (phenol soluble) and long chains (H_2O soluble) were isolated (40), indicating that prolonged reaction resulted in the synthesis of predominantly long chain polymer-protein adducts. Our results indicate that in S phase and after exposure to methylazoxymethanol acetate and benzamide both initiation and elongation rates increased simultaneously, consistent with an augmentation of the polymerase.

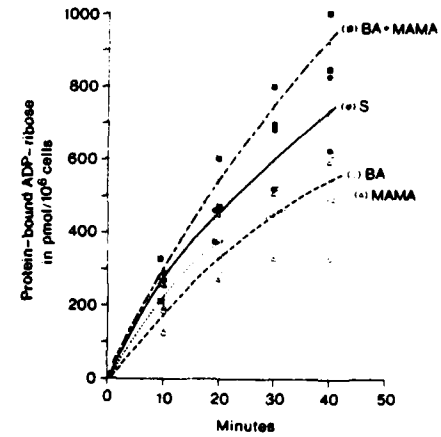

FIG. 5. Rates of poly(ADP-ribose) polymerase in synchronized human fibroblasts, assayed in the S phase. ●, S phase controls (S); ○, cells treated with benzamide (BA) (Table 2 and Fig. 4); △, 7.0 μM methylazoxymethanol acetate (MAMA); ■, simultaneous treatment with benzamide and the carcinogen (BA + MAMA). Assay conditions were the same as given for Table 2.

DISCUSSION

Application of nontoxic yet biologically effective concentrations of both carcinogens and benzamide tends to insure that results represent cellular biological mechanisms. The S-phase dependence of the effectivity of transforming agents and their antagonism by benzamide and the variation of poly(ADP-ribose) polymerase activity with the cell cycle (5, 39) suggest the participation of the macromolecular metabolism (i.e., induction, turnover) of the polymerase enzyme protein as a regulatory factor in poly(ADP-ribosyl)ation. Our results do not prove enzyme induction because only kinetics and products have been determined and more specific assays for the enzyme protein (e.g., by immunological methods) are required to settle this question. However, we have repeatedly found that an inhibitor of poly(ADP-ribose) polymerase, nicotinamide, at pharmacological doses sufficient to inhibit the enzyme *in vitro* can induce a

Table 2. Distribution and quantities of total, phenol-soluble, and H_2O-soluble protein-poly(ADP-ribose) adducts

Experimental conditions	Time of reaction, min	Protein-bound ADP-ribose, pmol per 10^6 cells		
		Total	Phenol soluble	H_2O soluble
G_1 block	20	203	45	157
	40	324	35	289
S phase	20	413	126	287
	40	868	13	854
+ Benzamide	20	207	67	140
	40	294	29	265
+ Methylazoxymethanol acetate	20	279	63	214
	40	348	19	329
+ Benzamide + methylazoxymethanol acetate	20	564	245	318
	40	905	15	888

Permeabilized cells (7.5×10^6; equivalent to ≈2 mg of protein) were incubated in a total volume of 500 μl containing 100 mM Tris chloride (pH 8.0), 10 mM EDTA, 20 mM $CaCl_2$, 0.5 mM NAD [^{14}C-labeled in the adenine moiety (26,000 dpm/nmol)], and 0.1 mM phenylmethanesulfonyl fluoride at 25°C. After 20 or 40 min the reaction was terminated by addition of 0.5 ml of 20% HClO at 0°C, and poly(ADP-ribosyl)ated proteins were separated (40). Each value is the mean of three analyses with a SD of ±20%.

variety of enzymes *in vivo* (41, 42); thus, the hypothetical mechanism proposed for benzamide is not without precedent. Based on the unique structural features of poly(ADP-ribose) (14, 15), it may be assumed that a structural regulation of physiological chromatin function in S phase has been reestablished by the augmentation of certain DNA-associated poly(ADP-ribose)-nonhistone-protein adducts and this process may be causally related to the prevention of transformation. A chromatin structure-dependent control of gene regulation has been proposed earlier (43), although molecular mechanisms involved were not identified. Currently held mechanisms of neoplastic transformation by oncogene expression (44–46), gene translocations, and amplifications (47, 48) may be extended by the poly(ADP-ribosyl)ation-dependent supramolecular control of availability of DNA domains that could involve critical enhancing regions.

It was shown that 3-aminobenzamide at high doses (300–600 mg/kg) if administered to rats, 4 hr after *in vivo* pretreatment with a hepatocarcinogen, appears to augment the development of premalignant liver foci, characterized by increased γ-glutamyl transpeptidase (49). An overwhelming dose of an inhibitor of poly(ADP-ribose) polymerase when administered *in vivo* is likely to serve primarily as an enzyme inhibitor of the polymerase and not as an inducer and therefore may reinforce carcinogenicity, especially if the carcinogen has been given prior to the inhibitor. It follows that specific pharmacokinetic and cellular kinetic conditions have to be observed to reproduce the antitransforming effect of nontoxic doses of inhibitors of poly(ADP-ribose) polymerase in intact animals. A variety of inhibitors of poly(ADP-ribose) polymerase have been found to serve as antitransforming agents in fibroblast cultures if applied under conditions described here (unpublished data); therefore, the observed effect is not confined to benzamide.

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BIOCHEMICAL BASIS OF THE REGULATORY ROLE OF POLYADENOSINE DIPHOSPHORIBOSE

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INTRODUCTION

Earlier developments in the field of poly (ADP-ribose) have been reviewed (1–4). Instead of a reappraisal of this often contradictory field the purpose of the present paper is to deal with experimental evidence obtained in this laboratory that may be the basis of the biochemical function of this polymer in physiologically functioning cellular systems. This necessarily selective approach was chosen because the compilation of highly complex results provided by numerous laboratories does not lend itself to the formulation of a convincing working hypothesis that explains the physiological function of poly (ADP-ribose). It may even be argued that poly (ADP-ribose) could be a metabolic curiosity with no physiological function except serving as an obligatory macromolecular catabolic product of NAD (6). That many aspects of the molecular biology of nucleic acids, especially in selected *in vitro* systems, can be profitably pursued without paying attention to the third type of nuclear polymer, poly (ADP-ribose), further tends to strengthen scepticism.

Against this relatively bleak background it should be mentioned that isolation of catalytic proteins that appear to participate in the biosynthesis and degradation of poly (ADP-ribose) (cf. 2) and the establishment of the elements of the primary structure of the polymer (cf. 5) are firm bases for future developments. Even the first stages beyond these basic accomplishments are fraught with uncertainties. Numerous laboratories report that histones, the most abundant nuclear proteins, are readily poly (ADP-)ribosylated both *in vitro* and *in vivo*, and yet the reason[illegible] these at best semiquantitative results are ambiguous. It could be assumed, for

example, that conventional analytical methods fail to detect the instability of polymer — non histone protein bonds and the apparent prevalence of histone polymer adducts merely indicates a survival of the more stable polymer — histone adducts. Yet formulation of a physiological function of poly (ADP–ribose) as a nucleoprotein modifying reagent critically depends on the nature of the polymer acceptor proteins.

The approach followed in our work predicts the subjects to be dealt with in the present paper. Biochemical studies will be presented that identify by various analytical methods the most probable types of nuclear proteins that serve as polymer acceptors in cellular and nuclear systems. Since the uniqueness of poly (ADP–ribose) as a protein modifier is its macromolecular nature, we further investigated the question whether or not this polymer has an ordered structure or merely exhibits a random structure as is the case with many polysaccharides. We subsequently studied the kinetics of polymerization and its reversal in a quasiphysiological model, the intact nucleus of permeabilized cells. The purpose of this kinetic experiment was the analysis of regulatory parameters of the polymerase and glycohydrolase enzymes in a nearly intact system. Finally, we have selected *in vivo* animal models and human cell culture systems for the demonstration of a physiological function of the protein-poly (ADP-ribose) system. By necessity the present paper represents a progress report in the areas listed, and results will be discussed by starting with structural aspects of poly (ADP-ribose) and then developed to areas of cell function.

Since the experimental work discussed in this paper deals with a variety of chemical and biochemical studies, no unified description of methods is feasible and therefore experimental procedures will be incorporated into each section.

CONFORMATION OF 2′ (5″–PHOSPHORIBOSYL)–5′–AMP

This nucleotide is the unique pyrophosphorolytic degradation product of the polymer. The α (1″ → 2) ribofuranosyl ribofuranoside bond (7–9) in 2′ (5″–phosphoribosyl)–5′–AMP) has been established by NMR studies. However, the descriptive structure of this nucleotide (Fig. 5 of ref. 5) as envisaged in an aqueous solution (8) does not predict its behaviour when the reactivity of the *cis*–diol with phenyl boronic acid is determined (10).

As shown in Figure 1, 2′ (5″-phosphoribosyl)-5′-AMP (PR-AMP) is not adsorbed on a phenylboronic acid column (Affigel 601) using 1 M ammonium carbonate as application buffer (pH 8.8) (A), whereas ADP–ribose behaves as expected and is eluted by 100 mM potassium phosphate (pH 4.0) as a single peak (B). Interference by electrostatic repulsion between negatively charged phosphate and boronate groups, a phenomenon that can occur at low ionic strength, was eliminated by the 1 M ammonium carbonate; therefore the

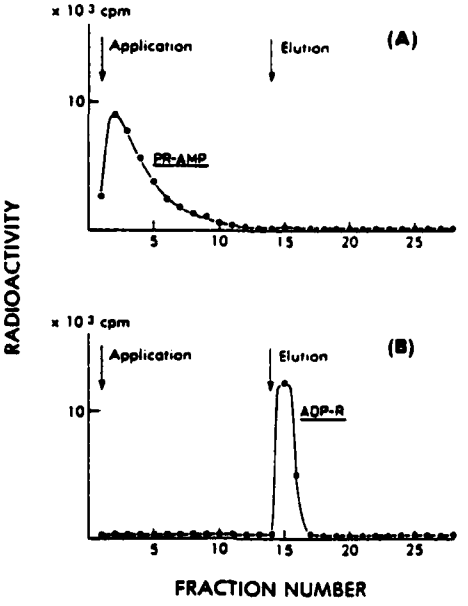

FIG. 1. Chromatographic behavior of PR-AMP (A) ADP-R (B) on boronate affinity resin. Application buffer was 1 M ammonium carbonate (pH 8.8) and elution buffer was 100 mM k-phosphate (pH 4.0). The change of buffers is indicated by arrows. Flow rate: 0.5 ml/min.

anomalous behavior of PR-AMP had to be ascribed to a structural property of this molecule.

The specificity of PR-AMP as compared to other *cis*-diol containing nucleotides with respect to boronate complexation is summarized in Table 1. When PR-AMP was dephosphorylated by alkaline phosphatase (EC 3.1.3.1)

TABLE I. BEHAVIOR OF ADENINE CONTAINING NUCLEOTIDES ON AFFIGEL 601

Nucleotides	Desorbed by the application buffer	Recovered by the elution buffer
Adenine	97.3%	0%
Adenosine	4.2%	89.0%
5'-AMP	4.6%	92.1%
5'-ADP	3.0%	91.3%
5'-ADP-R	1.2%	93.4%
NAD	2.0%	88.0%

Specificity of boronic acid gel towards adenine derivatives. Adenine derivatives were tested in the same application buffer as shown in Figure 1 (A) (1 M carbonate) to exclude possible interference by charge repulsions.

and dephosphorylation was confirmed by the absence of binding of ribosyl adenosine to AG 1 × 2 (compare Figures 2(A) and (B), the dephosphorylated product readily complexed with phenylboronic acid (Figure 2(C)). It is apparent that an interaction between the *cis*–diol with one of the phosphoric acid groups inhibits complexation with phenylboronate. Quantitative KIO_4 titration revealed the presence of a stoichiometric amount of *cis*–diol in PR–AMP (not shown, cf. 10); thus no anomaly exists with respect to the chemical entity of *cis*–diol. The molecular structure of PR–AMP was analyzed by a computer graphics model building system, utilizing available NMR data (8, 9). Eleven conformational parameters are required to specify the conformation of PR–AMP with fixed bond angles and lengths. Nine are due to single bond rotations and two to ribose puckering as was described in detail (10). If the *cis*–diol of ribose in the 5″–phosphoribosyl moiety were to form a boronate complex, it would be constrained to O_1'' *endo* or O_1'' *exo* conformation, and we found by graphic computation only the O_1'' *exo* to be

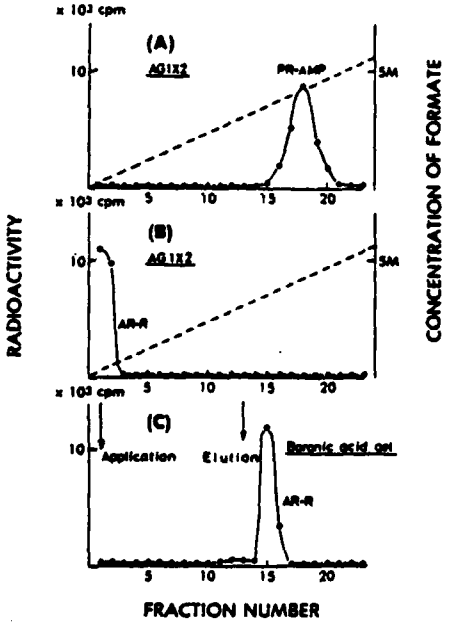

FIG. 2. Chromatographic behavior of PR–AMP and ribosyl-adenosine on AG 1 × 2 and boronic acid (Affigel 601) resins. (A) PR–AMP was charged on AG 1 × 2 formate resin and eluted with a linear gradient from H_2O to 6 M formic acid. (B) The eluted PR–AMP was lyophilized and dephosphorylated by alkaline phosphatase (E.C. 3.1.3.1) and charged on an AG 1 × 2 formate resin. The ribosyl-adenosine eluted without adsorption by the H_2O-formate gradient. (C) Ribosyladenosine obtained from B was charged on a boronate column and eluted as a single peak in the system described in the legend of Figure 1.

sterically favorable. In the ribose of AMP moiety NMR data indicate equal proportions of C_2' *endo* and C_3' *endo* puckerings. A major observation in our studies is that the puckerings of the sugars significantly alter the position of the phosphate groups in relation to the rest of the molecule. With C_3' *endo* puckering, the 5'-phosphate of the AMP moiety is distant from the hydroxyl (*cis*-diol) group of 5"-phosphoribosyl moiety, whereas with C_2' *endo* puckering the 5'-phosphate is located near the *cis*-diol. A model was built with C_2' *endo*-gg-*anti* for the AMP moiety and O_1' *exo-tg* for the 5"-phosphoribosyl moiety. The contacts between the non-bonded atoms were checked and relieved by rotating about the sugar-sugar bond. We find that only the phosphate of the AMP moiety can interact with the *cis*-diol group, as shown in Figure 3, and this explains the unavailability for phenyl boronate complexation. In the polymer the AMP moiety of PR-AMP is bonded as a pyrophosphate; thus the interaction seen in the subunit of the polymer (PR-AMP) is modified, making the *cis*-diol available for boronate complexation, as found experimentally (11). The possibility cannot be ruled out that the type of interaction observed with PR-AMP may explain that poly ADP-ribosylated peptides in SDS gel at pH 5.0 behave electrophoretically indistinguishably from polypeptides devoid of the polymer (12).

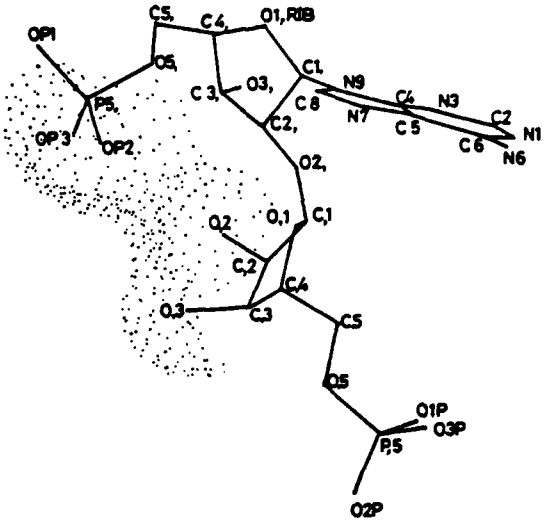

FIG. 3. Specific computer derived conformation of PR-AMP. The O atoms of the phosphate groups of AMP (OP_2, OP_3) and O atoms of *cis*-diol (0.2, 0.3) exhibit steric interaction. Computer modeling was performed on an Evan and Southerland Picture System PS 200 (Color Graphics) driven by a PDP 11/70 computer.

MACROMOLECULAR PROPERTIES OF POLY (ADP-RIBOSE)

In contrast to other covalent protein modifying reactions, e.g. phosphorylation, acetylation, methylation, etc., poly ADP-ribosylation involves the addition of a macromolecular component to proteins. If the polymer were of random structure as is known to be the case for many polysaccharides, interpretation of the biological significance of the protein modification process would tend to be in favor of protein transport. On the other hand, an organized, nucleic acid-like structure may help to explain protein-protein interactions or protein-DNA-binding, depending on the structural features of poly (ADP-ribose).

An experimental answer to this question was provided by the development of a relatively large scale (3–5 mg/batch) method for the isolation of uncontaminated polydisperse poly (ADP-ribose). The principle of the procedure consisted of extensive purification of enzymatically synthesized poly (ADP-ribose) on a boronate affinity column (13) followed by fractionation of oligomers with long ($n \approx 35$), medium ($n \approx 9$) and short ($n \approx 2$) oligomers. A correlation of spectral ($A_{280}/_{260}$, hypochromicity) and CD analyses with chain length was readily accomplished.

The effect of increasing concentration of phosphate on $A_{280}/_{260}$ of polydisperse poly (ADP-ribose) is illustrated in Figure 4. It is clear that $A_{280}/_{260}$ cannot be used as an optical test for purity of the polymer, as has been customarily done in the past, since the influence of increasing ionic strength on this spectral parameter suggests an effect on conformation. This is more explicitly shown in Figure 5. In the experiment described in Figure 5 both the

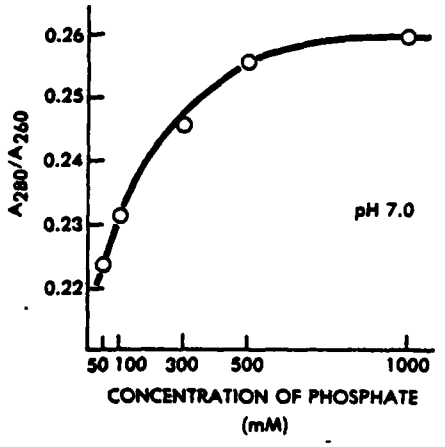

FIG. 4. The influence of concentration of phosphate on the $A_{280/260}$ of polydisperse poly (ADP-ribose) equivalent to 12.4 nmol/ml.

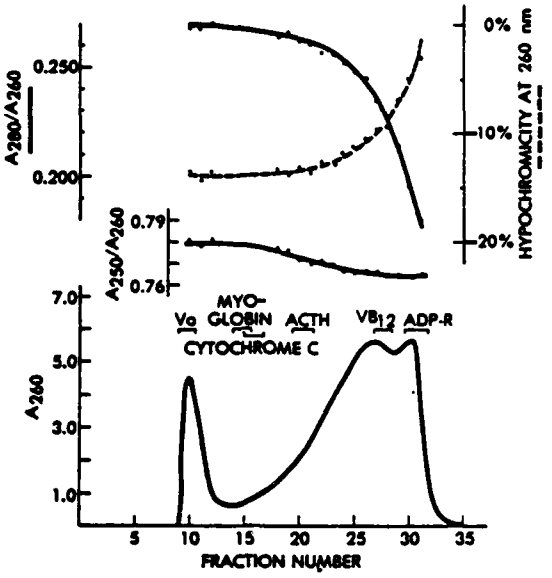

FIG. 5. Correlation between chain length and spectral properties of poly (ADP-ribose) determined at room temperature. The upper part illustrates spectral properties; left ordinate = $A_{280}/_{260}$; right ordinate = hypochromicity calculated from the specific radioactivity of PR-AMP prepared from the polymer. The lower part shows molecular filtration of the polydisperse polymer on Sephadex G-50 with molecular markers (cf. 17).

$A_{280}/_{260}$ and % hypochromicity were determined in poly (ADP-ribose) fractions of varying chain length. The upper part of Figure 5 depicts optical measurements and the lower portion illustrates the separation of polymers into fractions of varying chain length by molecular filtration. There was a significant increase in hypochromicity (from 0 to −14%) and a simultaneous increase in $A_{280}/_{260}$. Both responses reached a plateau at longer chain lengths.

The temperature dependence of CD spectra of long chain polymers is illustrated in Figure 6. At 5°C there are two large positive θ bands at 205 and 272.5 nm and one negative band at 249.5 nm with a small shoulder at 222–225 nm. At 75°C the θ signals diminished at 272.5 and 249.5 nm, giving place for a new negative band at 267.5 nm. The small shoulder seen at 5°C was enlarged into a negative band at 220 nm when temperature was raised to 75°C. At intermediate temperatures θ values assumed intermediary positions with two isosbestic points at 228 and 259 nm, exhibiting also a temperature increase dependent red shift (Figure 6, inset I) and a melting curve measured at 249.5 nm (Figure 6, inset II).

The temperature induced effects on CD spectra were also studied with medium and short chain oligomers. A temperature increase dependent red

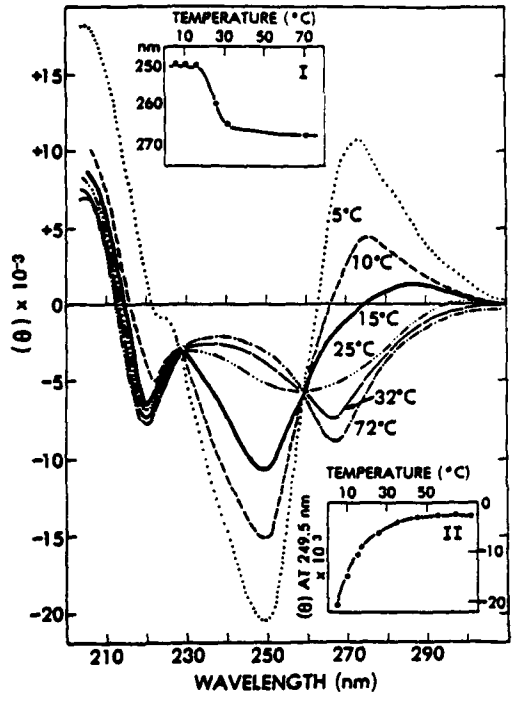

FIG. 6. The influence of increasing temperature on the CD spectrum of long chain poly (ADP-ribose), n = 35. Inset I = variation of ς in nm of θ_{max} (ordinate) at varying temperature, and inset II = meltin curve at ς 249 nm. CD was determined in a JASCO J-500A spectropolarimeter calibrated with D-10-camphorsulfonic acid and expressed as mean residue ellipticity.

shift was still seen with chains of medium length, whereas with short oligomers only the magnitude of θ exhibited temperature dependence. At 72°C the differences between CD spectra of all three species of oligomer vanished, indicating that heat denatured long and medium chains assumed the conformation of the short chain species (cf. 13).

The sum of these results strongly indicates that poly (ADP-ribose) has ordered conformation, and in fact at 5° the CD spectra resemble those of poly (A), poly (U), poly (A + U) and DNA, but not RNA because RNA has no major band around 245 nm (14–16). This similarity in conformation is remarkable in view of the significant differences in the molecular configuration between poly (ADP-ribose) and the mentioned nucleic acids. Interpretation of these observations depends on more precise structural studies (e.g. X-ray diffraction analyses).

It is concluded that high probability exists that the biological effect of

covalent modification of proteins on a molecular level depends on the interaction of this nucleic acid type structure with nucleic acids, probably DNA, conferring possible regulation of association of selective proteins with DNA.

THE NATURE OF ACCEPTOR PROTEINS IN THE CELL NUCLEUS

The prevalence of histones in the nucleus prompted earlier investigators to study poly ADP-ribosylation of these basic proteins both *in vivo* and *in vitro* (cf. 1, 2). Well developed techniques available for the isolation of histones provided technical advantages and probably convenience of this technology also propagated this experimental approach. Although the participation of non-histone proteins as polymer acceptors was realized in many laboratories, it was not until quantitative methods were applied that the contribution of both types of nuclear proteins as polymer acceptors could be assessed. We have employed quantitative immunochemical (17), affinity chromatography (11) and quantitative gel electrophoretic (12, 18) techniques for the resolution of this question. Results are summarized in Table 2.

TABLE 2. THE QUANTITATIVE ASSOCIATION OF POLY (ADP-RIBOSE) WITH NON-HISTONE PROTEINS

Experimental conditions and methods	% polymer bound to non-histone proteins
Immunochemistry $n > 4$ (rat liver)	99% (ref. 17)
Affinity chromatography (hamster liver)	86% (cf. 11)
Quantitative gel electrophoresis (cardiocyte nuclei and 9L-gliosarcoma cells)	80–85% (cf. 12, 18)

Although a definite fraction of poly (ADP-ribose) is consistently found to be bound to various histones (mostly H_1), this fraction is much smaller than that associated to non-histone proteins. Considering the large quantity of histones in nuclei the preferential non-histone protein association of the polymer becomes even more obvious. The participation of non-histone proteins in the regulation of differentiation and cell specificity of macromolecular metabolism is well recognized (cf. 19), although the underlying molecular mechanisms are yet largely unknown. It is for this reason that our attention became focussed on biological phenomena concerned with differentiation, such as the action of developmental hormones, ontogenic development and carcinogenesis, and we proceeded to study poly (ADP-ribose) metabolism in related models.

THE REGULATION OF SYNTHESIS OF POLY (ADP-RIBOSE) IN PERMEABILIZED RAT BRAIN 9L-GLIOSARCOMA CELLS

Before the discussion of results obtained in the area of differentiation and development it seems plausible to consider biochemical regulation of poly ADP-ribosylation in a single cell system. Identification of rate limiting processes in a simpler system may aid possible interpretations of more complex models.

Although purified proteins have been isolated that catalyze both the biosynthesis and hydrolytic cleavage of poly (ADP-ribose) (cf. 1, 2), the catalytic control especially of the polymerase enzyme is poorly understood. In the purified form the polymerase serves as its own polymer acceptor (20), and this automodification of the enzyme results in enzyme inhibition. It follows that prediction from *in vitro* kinetics of the purified enzyme to conditions that prevail in intact nuclei would be difficult because it can be assumed that *in situ* the polymerase will poly ADP-ribosylate a variety of proteins (see Table 2) before its autopoly ADP-ribosylation signals termination of the entire process. TransADP-ribosylation from the enzyme protein to histones has been ruled out experimentally (20); thus it has to be assumed that an interaction between the ADP-ribose donor (i.e. NAD), the polymerase and appropriate polymer initiation accepting proteins must take place under V_{max} conditions. The multisubstrate kinetics of this catalysis has so far not been considered, probably because of the extreme technical difficulties encountered in the isolation of an acceptor protein that — at least for a brief period — will eliminate autoinhibition of the reaction sequence. The polymerase is known to have a dual catalytic activity (20), one being equivalent to NAD glycohydrolase, producing ADP-ribose, and the second is the polymerase reaction that adds ADP-ribose to the ribose moiety of growing chains. It was also shown that free ADP-ribose readily forms protein adducts (21), and these adducts can serve as initiators for polymer chain formation (22). It is evident that under optimal enzymatic conditions that presumably prevail in intact cell nuclei, a reasonable catalytic process of initiation, elongation and termination of polymerization reaction exists. We have tested this mechanism in lysophosphatidyl choline treated rat brain 9L-gliosarcoma cells, which offer no permeability barrier to externally added macromolecules (proteins), yet maintain an essentially intact nuclear structure (cf. 18). When these permeabilized cell ghosts are incubated with DNA-ase I as an activator (for mechanism see below) and with 50 and 500 μM NAD as substrate and the chain length of polymers formed is determined after 3, 30 and 50 min reaction time, results shown in Figures 7 and 8 are obtained.

It is clear that with 50 μM NAD there is rapid initiation as well as elongation (Figure 7A), followed by rapid decay of large polymers but a sustained rate of initiation. With 500 μM NAD as substrate there is a seemingly more regular initiation process and elongation taking place, and no precipitous decay of

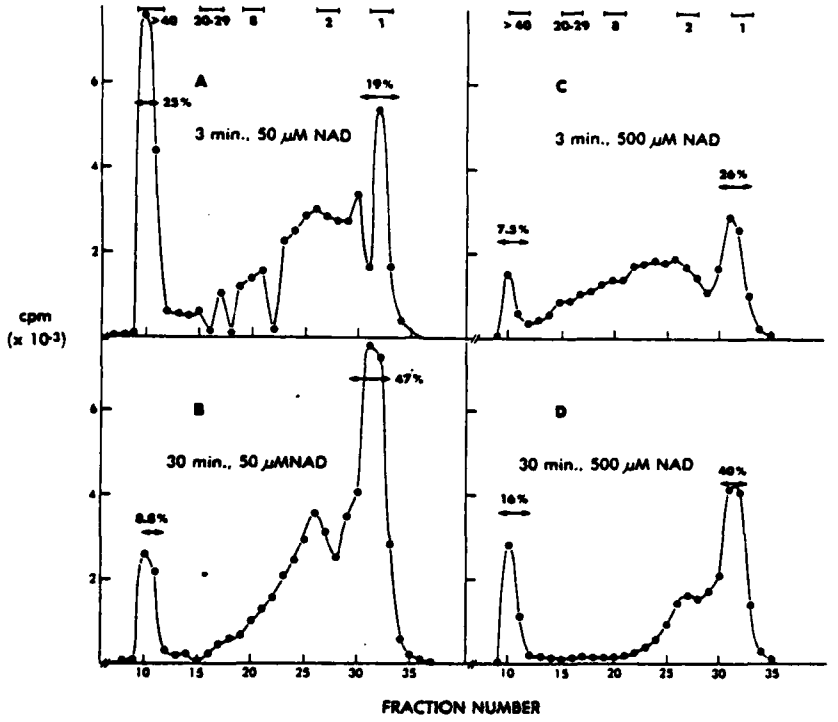

FIG. 7. Time dependent variation of the chain length of polymers formed in the presence of 50 (A,B) and 500 (C,D) μM NAD by 250 μg (protein) 9L-gliosarcoma cell ghosts for 3 or 30 min (25°C, conditions described in ref. 18). The chain length of the protein free polymer, obtained by base hydrolysis, was determined by molecular filtration on Sephadex G-50; elution system: I, NaCl, 01 M Tris-HCl, pH 7.0, flow rate 12 ml/h, fraction vol. = 4 ml.

long chains is seen. With the passage of time the polymers are grouped around long chains only (Figure 8), apparently consistent with a state where all initiator sites are saturated and only elongation takes place until presumably automodification of the polymerase terminates the entire process. It is evident that the intact nuclear system behaves like a predictable kinetic model for polymerization and thus is suitable for mathematical analysis following known principles of the process of polymerization (23). Data from Figure 8 were replotted in Figure 9 in order to compare the weight fraction of chain length (n) to one plausible reference form, the Poisson distribution (cf. 23) Assuming a linear relationship between chain length and fraction number, an assumption based on experimental data (17), a weight fraction maximum $W_{n\,max}$ was obtained. The points in Figure 9 represent the ratio $W_n / W_{n\,max}$ as

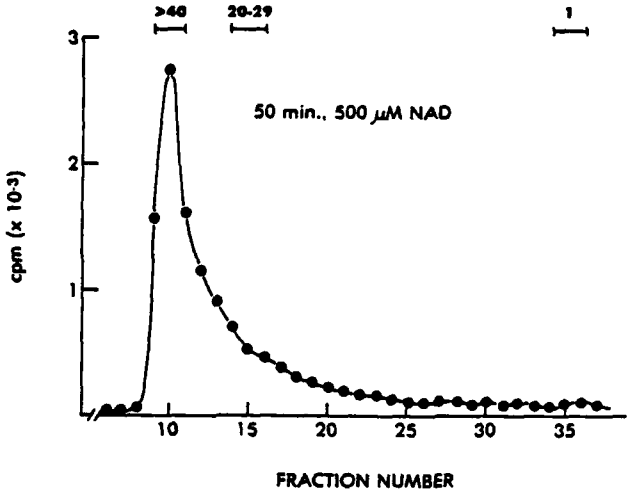

FIG. 8. The chain length distribution near equilibrium. Conditions were the same as described in Legend of Figure 7, except t = 50 min and NAD = 500 μM.

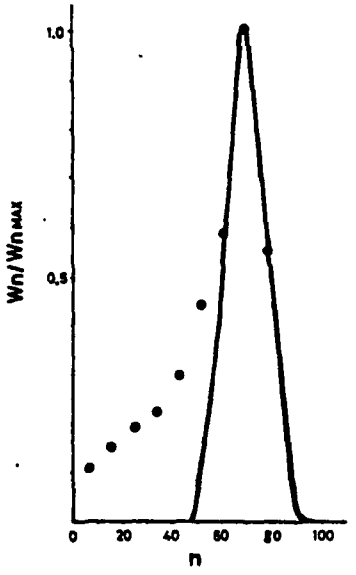

FIG. 9. Poisson distribution (solid line) of polymers calculated from results shown in Figure 8. The dots are experimental values; the solid line is the result of computation as described in the text.

ordinate against chain length (n) and the solid curve is the calculated value expected from the Poisson distribution:

$$W_n / W_{n\,max} \approx (\gamma/n)^{n+1/2} \exp(n-\gamma)$$

with $\gamma = 70$. The factorial n (n!) has been replaced by the Stirling's approximation for arbitrary n and for $n = \gamma$ (24). The experimental points on either side of the maximum are in close conformity with this calculation, but for shorter chain length there is increasing deviation from the sharply peaked reference curve. The most probable explanation for this deviation is the difference of reactivity of initiation sites. Once the chains are initiated and surpass some minimum size their growth rates should be invariant and no longer reflect the point of origin. It follows that the most likely control site of polymerization is at the initiation sites.

Independent experimental evidence confirms this hypothesis. It follows from the ADP-ribose-protein adduct formation (21) that nucleophiles should compete for the initiation reaction of polymerization. This is found experimentally, as summarized in Table 3.

TABLE 3. INHIBITION OF PROTEIN-POLYADENOSINE DIPHOSPHORIBOSYLATION IN PERMEABILIZED 9L-GLIOSARCOMA CELL GHOSTS BY BASIC AMINO ACIDS

No.	Experimental conditions	ADP-R incorporated nmol/mg protein/5 min	% Inhibition
1	Control, no DNA-ase I 5 min incubation	0.49	
2	Control, DNA-ase I 200 ug/ml 5 min incubation	5.25	
3	As 1 + 50 mM arginine	0.40	18
4	As 2 + 50 mM arginine	2.71	48
5	As 1 + 50 mM L-lysine	0.35	29
6	As 2 + 50 mM L-lysine	3.01	43
7	As 1 + 50 mM arginine-methyl ester	0.16	68
8	As 2 + 50 mM arginine-methyl ester	0.53	90

THE MECHANISM OF ACTION OF DNA-ase I PROTEIN (E.C. 3.1.4.5) ON POLY (ADP-RIBOSE) POLYMERIZATION IN INTACT NUCLEI

It has been known for some time that addition of DNA-ase I to permeabilized cells increases poly (ADP-ribose) polymerase to a maximal level (cf. 3, 4). It was assumed that the nucleolytic action of DNA-ase I on DNA in some way activates the polymerase and an elaborate hypothesis was proposed that professed a regulatory role of DNA fragmentation on poly

(ADP-ribose) synthesis (cf. 3). We found no such relationship in two dissimilar *in vivo* models (11, 23) between DNA size and poly (ADP-ribose) polymerase activity and proceeded to investigate the mode of action of DNA-ase I in permeabilized 9L-gliosarcoma cells (18). The experimental approach included the labeling of cellular DNA with [^{3}H] thymidine, resulting in DNA of very high specific activity (30,000 dpm/μg DNA) permitting the determination of the quantity and molecular size of DNA with very great accuracy. Since it is well known that DNA-ase I activity can be varied at will by experimental conditions, we studied the effects of active, inhibited and base denatured DNA-ase I on DNA molecular size, quantity and its effect on poly (ADP-ribose) polymerase under a wide variety of conditions. As illustrated in Table 4, both enzymatically active DNA-ase I and Ca^{2+} depleted DNA-ase I,

TABLE 4. THE EFFECT OF ADDED DNA-ase I ON THE MOLECULAR SIZE AND CELLULAR CONCENTRATION OF DNA AND ON POLYADENOSINE DIPHOSPHORIBOSE POLYMERASE ACTIVITY OF PERMEABILIZED 9L-GLIOSARCOMA CELL GHOSTS

No.	Experimental conditions	DNA size in Svedberg units (S)	DNA concentration: (μg DNA/mg cell protein)	Polyadenosine diphosphoribose polymerase (nmol ADP-R incorporated in 5 min/mg protein)
1	Gliosarcoma cells	59.0	25.0	0.6
2	1 + 100 μg/ml DNA-ase I + Ca^{2+} + NAD added simultaneously	not measurable after 5 min incubation	0.3	7.7
3	1 + preincubation with DNA-ase I for 10 min	not measurable after 5 min incubation	0.3	6.7
4	1 + preincubation with DNA-ase I for 20 min	not measurable after 5 min incubation	0.3	6.4
5	1 + Ca^{2+} depleted DNA-ase I (200 μg/ml) + Mg^{2+} added simultaneously	28.0	24.0	5.7
6	1 + preincubation with Ca^{2+} depleted DNA-ase I for 5 min	28.0	24.0	6.2
7	1 + preincubation with Ca^{2+} depleted DNA-ase I for 10 min	28.0	24.0	6.6
8	1 + preincubation with Ca^{2+} depleted DNA-ase I for 20 min	28.0	24.0	7.2

In Exps. 3, 4, 6, 7 and 8 the reaction was started by adding [^{14}C]-NAD.

which retained only the capability to produce a single strand break, halving the molecular size of DNA, maximally activate poly (ADP-ribose) polymerase; therefore no correlation between the magnitude of DNA breakdown (which was 98.5% in the case of active DNA-ase I) and poly (ADP-ribose) polymerase could be established. Furthermore, base denatured DNA-ase I protein also increases the polymerase activity and conditions were found where increased poly (ADP-ribose) polymerase activity actually coincided with an inhibition of DNA breakdown by endogenous nucleases (cf. 18). The effect of DNA-ase I on poly (ADP-ribose) polymerase activity was inhibited by actin, which is known to bind DNA-ase I (cf. 18). The most probable mode of action of DNA-ase I on the polymerase appeared to be mediated by the binding of DNA-ase I with nuclear actin, resulting in a nuclear conformation change that makes more initiating protein (mostly non-histone) sites available for polymer initiation. The observed kinetics of polymer initiation, elongation and termination (Figures 7 and 8) is consistent with this conformation dependent mechanism although molecular details are still lacking. However, the DNA-fragmentation dependent activation of the poly (ADP-ribose) polymerase could not be confirmed and it is likely that coincidence of phenomena that bear no causal relationship have been correlated in the past (3). It is of interest that DNA-ase I protein also serves as a polymer acceptor in the 9L-gliosarcoma system (18). The kinetics of degradation of intranuclearly synthesized poly (ADP-ribose) is also regulated by chromatin structural factors (18), since at high polymer concentration decay is minimal or completely absent. This phenomenon is best explained by product inhibition of glycohydrolase by ADP-ribose, an effect far greater when the polymer is formed *in situ* than when added externally (cf. 18).

REGULATION OF POLY (ADP-RIBOSE) BY Mg^{2+}, Ca^{2+} AND POLYAMINES

The 9L-gliosarcoma cell ghost model served a particularly useful purpose in the exploration of regulation of poly (ADP-ribose) polymerase activity by bivalent cations and polyamines (18). When all endogenous nuclear bivalent cations were chelated by EDTA the polymer synthesis proceeded at a slow rate but surprisingly the macromolecular profile of polymers synthesized did not differ significantly from systems containing Mg^{2+} as an activator (Figures 7 and 8). This demonstrates that neither initiation nor elongation requires bivalent cations, and it seems that activation by bivalent cations affects in an as yet unknown manner the availability of initiation sites. The influence of Ca^{2+}, Mg^{2+} and polyamines is summarized in Table 5. It is evident that without the catalytic effect of enzymatically inactive DNA-ase I Ca^{2+}, Mg^{+}, spermidine and spermine increase rates and Ca^{2+} is a more active cation than

TABLE 5. EFFECTS OF Ca^{2+}, Mg^{2+} AND POLYAMINES ON POLYADENOSINE DIPHOSPHORIBOSYLATION OF PROTEINS IN 9L-GLIOSARCOMA CELL GHOSTS IN PRESENCE AND ABSENCE OF DNA-ase I

No.	Addition to basic incubation mixture	ADP-R incorporated (nmol/mg protein per 30 min)	
		at 50 μM NAD	at 500 μM NAD
1	—	0.11	0.68
2	Pu	0.11	0.69
3	Spd	0.29	1.26
4	Sp	0.30	1.41
5	Ca^{2+}	0.42	2.20
6	Ca^{2+} + Pu	0.44	2.04
7	Ca^{2+} + Spd	0.48	2.30
8	Ca^{2+} + Sp	0.20	1.60
9	Mg^{2+}	0.27	1.10
10	Mg^{2+} + Pu	0.22	1.21
11	Mg^{2+} + Spd	0.28	1.39
12	Mg^{2+} + Sp	0.20	1.11
13	DNA-ase I	0.11	0.52
14	DNA-ase I + Pu	0.12	0.62
15	DNA-ase I + Spd	0.31	1.12
16	DNA-ase I + Sp	0.30	1.01
17	DNA-ase I + Ca^{2+}	1.96	7.15
18	DNA-ase I + Ca^{2+} + Pu	1.85	7.98
19	DNA-ase I + Ca^{2+} + Spd	2.89	13.11
20	DN-ase I + Ca^{2+} + Sp	1.90	7.12
21	DNA-ase I + Mg^{2+}	1.15	5.83
22	DNA-ase I + Mg^{2+} + Pu	1.38	5.83
23	DNA-ase I + Mg^{2+} + Spd	1.33	6.44
24	DNA-ase I + Mg^{2+} + Sp	0.54	4.15

Pu = putrescine; Spd = spermidine; Sp = spermine.

Mg^{2+}. The effects are similar but on a larger scale with DNA-ase I present, and it is clear that the catalytic effect of DNA-ase I itself requires either Ca^{2+}, Mg^{2+}, spermidine or spermine. Combination of Ca^{2+} and spermidine specifically augmented poly (ADP-ribose) polymerase activity (Exp. 19), indicating that under V_{max} conditions (i.e. in the presence of a catalytic protein like DNA-ase I) Ca^{2+} and spermidine may be the real cellular regulators of the polymerase. This observation has probable physiological implications. Animal cells contain mM concentrations of Ca^{2+} as well as spermidine. Intracellular distribution changes of Ca^{2+} and the metabolism of spermidine are under the control of numerous well known regulatory systems. Therefore seemingly distant biochemical variations can through Ca^{2+} or spermidine effect large changes in poly ADP-ribosylation rates without the necessity of altering enzyme content of nuclei. Exploration of these regulatory systems is a subject of current research.

EXPERIMENTAL EVIDENCE INDICATING A BIOLOGICAL FUNCTION OF POLY ADP-RIBOSYLATION IN ANIMAL MODELS

It was primarily the recognition that nuclear non-histone proteins are the most significant polymer acceptors that led to studies concerned with the action of developmental hormones and carcinogenesis on poly ADP-ribosylation. The first specific response related to differentiation and development was seen in cardiocyte nuclei (25) where a ten-fold change of poly ADP-ribosylation was recorded as a function of a three week period of postnatal development. A similar cell type specific and age dependent inhibition of poly ADP-ribosylation by hydrocortisone and aldosterone was also described (26). The sterol hormones, besides inhibiting synthesis of the polymer, also activate its catabolism; thus a dual effect resulting in the loss of the modifying homopolymer from presumably regulatory nuclear proteins is effected. The hormonal influence studied most extensively thus far is the effect of T_3 on RNA synthesis (12, 27). In this model the mode of action of poly ADP-ribosylation on RNA synthesis *in vitro* was demonstrated with isolated cardiocyte nuclei, as shown in Table 6. Poly ADP-ribosylation *in vitro* was

TABLE 6. THE EFFECT OF *IN VITRO* POLY ADP-RIBOSYLATION ON RNA SYNTHESIS *IN VITRO* BY CARDIOCYTE NUCLEI

Experimental conditions	RNA synthesis (nmol UMP incorporated per 80 μg nuclear protein in 10 min
1 Nuclei of controls	2.6
2 Nuclei of controls after poly ADP-ribosylation	2.7
3 Nuclei from T_3 treated rats	3.7
4 3 after poly ADP-ribosylation	0.7
5 4 in the presence of 20 mM nicotinamide	3.4

accomplished by preincubation of cardiocyte nuclei with NAD for 30 min (cf. 12). It is clear that pretreatment with T_3, which induced a large increase in cardiac ventricular size (12, 27), was a prerequisite for the large inhibitory effect of poly ADP-ribosylation on RNA synthesis. This is the first clear indication of the regulatory influence of poly ADP-ribosylation on a major enzymatic process involved in nucleic acid metabolism. The molecular mechanism of this inhibition is being studied presently. Gel electrophoretic isolation of poly ADP-ribosylated polypeptides indicates that the *in vivo* treatment of rats with T_3 causes an uneven inhibition of poly ADP-ribosylation of certain groups of cardiocyte polypeptides with a molecular mass of 130, 80–90, 65–80, 55–65 kd, where the decrease ranges from 70–75%, whereas the diminution of poly ADP-ribosylation in other groups of peptides is between 15 and 59%. These results are shown in Table 7. Preincubation with T_3 at 10^{-7} M inhibits *in vitro* poly ADP-ribosylation in cardiocyte nuclei, and a

similar *in vitro* effect was observed with steroid hormones. The probable role of poly ADP-ribosylation dependent inhibition of RNA physiological synthesis is the termination of hormone (stress) induced hypertrophy (cf. 12).

TABLE 7. DIFFERENTIAL EFFECTS OF T_3 TREATMENT ON THE DECREASE OF *IN VITRO* POLYADENOSINE DIPHOSPHORIBOSYLATION OF CARDIOCYTE NUCLEAR POLYPEPTIDES SEPARATED BY GEL ELECTROPHORESIS

Polypeptides No.	Molecular mass (kd)	Specific radioactivity (cpm/ weight of peak area) saline treated	T_3 treated	% change
1	240	456	317	-30.5
2	210	36	30	-16.7
3	180	90	49	-46.0
4	180–130	394	261	-33.8
5	130	666	149	-77.6
6	120	243	105	-56.8
7	113–90	334	200	-40.1
8	90–80	302	89	-70.5
9	80–65	360	94	-73.9
10	65–55	227	73	-67.9
11	40	43	24	-44.4
12	37–27	147	80	-45.6
13	27–18	67	56	-16.4
14	18	163	66	-59.5

CARCINOGENESIS

There is a large increase in poly ADP-ribosylation of nuclear proteins in the early precancerous hamster liver, as demonstrated by *in vivo* labeling of the polymer followed by the isolation of polymer protein adducts with the aid of affinity chromatography (cf. 11). The polymers and proteins were dissociated after the isolation of adducts and the molecular mass of both protein free polymers and polymer free proteins determined by HPLC molecular filtration. The increase of poly ADP-ribosylation of largely non-histone proteins of larger molecular mass (between 150 and 210 kd) was specific for the precancerous state and the opposite, a decrease of poly ADP-ribosylation, was found during growth hormone induced growth (28). By *in vivo* labeling a crossover was observed at the poly (ADP-ribose) level during growth hormone induced growth (28), whereas this crossover occurred at the NAD level at the precancerous state (11).

On the basis of these results the question was posed whether or not inhibition of poly ADP-ribose polymerase *in vivo* can inhibit the carcinogenic process. It is noteworthy that the augmentation of poly ADP-ribosylation seen in the precancerous state was more specifically found to coincide with a promoter process, i.e. liver regeneration, even without carcinogen treatment (29). Consequently a cellular model of carcinogenesis was selected where the temporal events of carcinogenesis were experimentally

defined (30). Human fibroblasts synchronized by nutritionally imposed G_1 block followed by release and stimulation, when exposed to ultimate carcinogens immediately after G_1 block, respond with transformation to cells that exhibit anchorage independent growth and tumor formation in animals that have been injected with the transformed cells.

It is of considerable interest that the above described synchronization method, which is a prerequisite of *in vitro* carcinogenesis with human fibroblasts, also induces a 4- to 5-fold increase of rates of protein-poly ADP-ribosylation as assayed by the cell permeabilization technique of (cf. 18). These results are shown in Table 8.

TABLE 8. INCREASE OF POLY ADP-RIBOSYLATION IN SYNCHRONIZED AND G_1 BLOCK RELEASED HUMAN FIBROBLASTS AS COMPARED TO RANDOMLY GROWING CELL CULTURES

No.	Growth conditions	Assay conditions	poly ADP-ribosylation (nmol ADP-ribose/ mg protein in 10 min)	Activation ratio by DNA-ase I
1	Random	(a) no DNA-ase I	0.33	7.1
		(b) + DNA-ase I	2.36	
2	Synchronized and G_1 block released (cf. 30)	(a) no DNA-ase I	1.40	1.91
		(b) + DN-ase I	2.67	

Each assay contained 260 μg (protein) permeabilized cells, about 10^6 cells, 500 μM NAD, Mg^{2+} (10 mM) and buffer as described (18). Inactivated DNA-ase I, when added, was present in a concentration of 200 μg/ml.

The significant increase of poly-ribosylation was measurable only when endogenous polymer acceptors were assayed (1a and 2a). According to our interpretation this would be explained by a conformational change of chromatin in synchronized cells, allowing the increased availability of initiator sites. When DNA-ase I protein is added the difference disappears between random and synchronized cells, indicating that under V_{max} conditions, when presumably all acceptor sites are available, the enzymatic rate differences vanish. We interpret these results to indicate that rate changes are not related to enzyme content but to conformationally induced release of ADP-ribose acceptor sites. There is increasing evidence accumulating that favors change of chromatin conformation, probably at the supramolecular level, as a significant mechanism of gene regulation (31) and recently electron microscopy suggested a similar structure related regulation of poly ADP-ribosylation (32).

The most significant aspect of results shown in Table 8 is the similarity between increased poly ADP-ribosylation in the precancerous state *in vivo* (11) and the appearance of the same phenomenon in cell cultures predisposed to be susceptible to carcinogenesis.

As summarized in Table 9, exposure of human fibroblasts to benzamide, a highly potent inhibitor of poly ADP-ribose polymerase in cells (cf. 18), at a time schedule that coincides with the activation of this enzyme system by G_1

TABLE 9. PREVENTION OF CARCINOGENESIS IN HUMAN FIBROBLASTS BY BENZAMIDE, A SPECIFIC INHIBITOR OF POLY (ADP-RIBOSE) POLYMERASE

No.	Treatment of cells	No. of cancer cell colonies formed per 50,000 cells
1	Methylazoxy-methanol-acetate	300–350
2	1 + benzamide	1
3	N-me-N-nitrosoguanidine	45–50
4	3 + benzamide	1
5	3-hydroxy-1-propane-sulfonic acid γ-propiolactone	40–42
6	5 + benzamide	1
7	Benzamide	1

block and release completely abolishes the carcinogenic effect of a variety of ultimate carcinogens. The concentration of externally added benzamide was 1 mM, but by tracer methodology it was shown that only 0.7–1.5% of benzamide enters the cells, and the calculated nuclear concentration (between 8 and 10 μM) is exactly in the range where poly ADP-ribose polymerase is selectively inhibited 40–50% by this non-toxic agent. Benzamide is recovered without metabolic conversion from human fibroblasts following exposure for 18 hr, as determined by HPLC analysis.

The molecular mechanism of this carcinogenesis prevention by the inhibition of poly (ADP-ribose) polymerase is the subject of further studies (33); however, the phenomenon itself leaves little doubt regarding a real cellular regulatory function of poly (ADP-ribose). Artefacts such as a reaction of carcinogens with benzamide are readily ruled out on a chemical basis and the selectivity of the observed intervention appears to be restricted to the polymerase reaction.

SUMMARY AND PROSPECTUS

Poly ADP-ribosylation of nuclear proteins does not seem to relate to any recognizable metabolic reaction in cellular economy except that the polymer is an obligatory catabolic product of NAD. The turnover of NAD is regulated by the metabolism of the polymer. It is the apparently elusive nature of this covalent protein modification that appears to sustain an aura of uncertainty related to its cellular significance.

The experimental evidence discussed in this report follows a relatively uncomplicated line of reasoning. It was shown that the polymer possesses a highly significant secondary structure (reminiscent of DNA); thus its role as a macromolecular association promoting or regulating polymer within the

nucleus is feasible. It follows from this physico-chemical consideration that a physiological role has to be identified in the specific field of regulation, not as an essential metabolic component of macromolecular metabolism. Based on the prevalent targets of poly ADP-ribosylation, non-histone proteins, the latter known to play a specialized function in differentiation and development, we identified indeed signals of poly ADP-ribosylations that correlate to age, differentiation, and are developmental hormone mechanisms and significant in carcinogenesis. The sum of this evidence justified more detailed molecular studies, and these are concerned with the identification of highly important regulatory nuclear proteins for which poly (ADP-ribose) can serve as an identifying marker. Isolation of these poly ADP-ribosylation susceptible non-histone proteins and their mechanism of binding to selective DNA segments (templates), thereby presumably influencing transcription selectivity, appears to us the most profitable future trend of research in this relatively undeveloped field.

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SYNTHESIS OF ^{14}C-LABELLED METHYLAZOXYMETHANOL ACETATE OF HIGH SPECIFIC ACTIVITY

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SUMMARY

The micro-scale synthesis of ^{14}C-labelled methylazoxymethanol acetate (^{14}C-MAM-acetate) having a specific activity of 110 mCi/mmol was accomplished in 25% yield from N,N'-[methyl-^{14}C]-dimethylhydrazine with a specific activity of 112.5mCi/mmol.

Key Words: Carbon-14; Methylazoxymethanol Acetate; Azomethane; Azoxymethane; Bromoazoxymethane; N,N'-dimethylhydrazine.

INTRODUCTION

Cycasin (β-D-glucosyl-azoxymethane)[1,2] is hepatotoxic and carcinogenic in rats[3,4] and this toxicity is attributable to the aglycone, methylazoxymethanol (MAM)[5,6], which is also a metabolite of 1,2-dimethylhydrazine. MAM-acetate has increased stability, reduced volatility, retains toxicity[7,8] and is a very potent transformer of human fibroblasts in culture (manucripts in preparation). Although the synthesis of ^{14}C-MAM-acetate was reported by Horisberger and Matsumoto[9], their synthesis of this compound, starting with commercially prepared ^{14}C-labelled azomethane, yielded only small quantities of labelled product. It was suggested that poor yields may reflect autoradiolysis. In contrast, we report here the micro-scale synthesis of ^{14}C-MAM-acetate of near theoretical specific activity and in significantly higher yields.

DISCUSSION

Using a modification of the synthetic scheme reported by Horisberger and Matsumoto[9] the synthesis of ^{14}C-MAM-acetate (5) was accomplished by oxidation of

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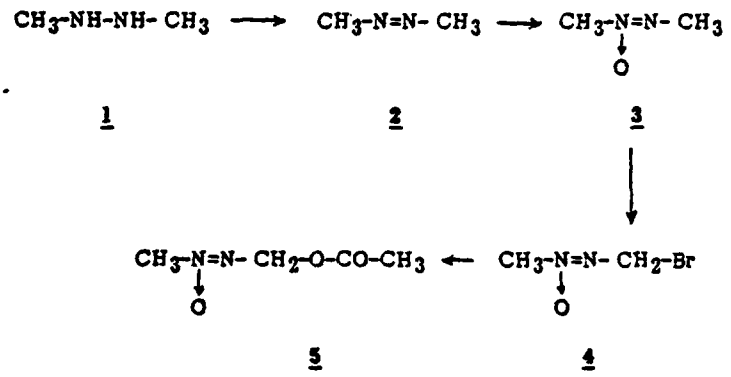
N,N'-[methyl-^{14}C]-dimethylhydrazine (1) to ^{14}C-azomethane (2) and subsequently ^{14}C-azoxymethane (3). Allylic type bromination followed by reaction with silver acetate afforded 5.



Although reaction conditions using cold material were optimized at each step, the reaction conditions for the radiolabelled material required considerable modification. Micro-scale production of high specific activity 5 was carried out using the described apparatus. The 25 ml reaction flask and 10 ml traps were critical; poor yields of 3 were obtained with unlabelled material on the micro-scale when these vessels were 50 and 25 ml, respectively.

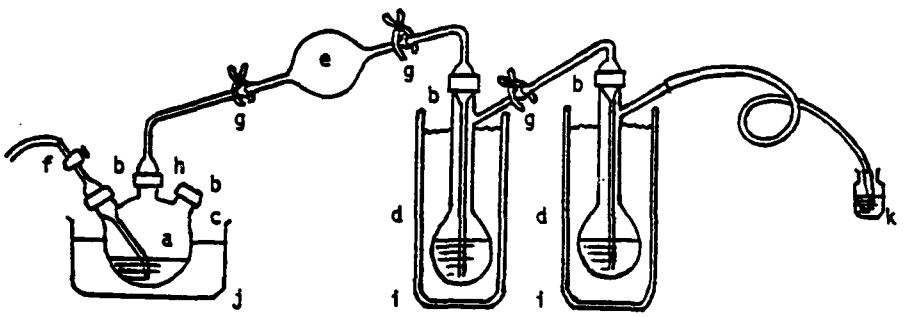
Under these conditions 1 was converted to 3 in 80% yield; a significant improvement over the 31% average reported by Horisberger and Matsumoto[9]. The argon flow rate of 5-6 ml/min is critical since a slower rate does not adequately carry the azomethane from the reaction flask and at faster flow rates azomethane escapes from the traps. Both traps were charged with the less polar methylene chloride solvent substituted for previously used ether[9]. Thus, the m-chloroperbenzoic acid is retained on the column during chromatographic purification on basic alumina.

The desirable temperature of trap #1 is 10 - 12°C. Too low a temperature results in precipitation of m-chloroperbenzoic acid and this in turn decreases conversion of azomethane (2) to azoxymethane (3). Gas flow and evolution of radioactive gas was routinely monitored by bubbling the trap effluent through scintillation cocktail. During 20 - 30 mCi reactions only 3 - 10 μCi of ^{14}C could be detected in the scintillation vial. A

significant difference in reaction time was required for bromination using labelled vs. unlabelled azoxymethane. For unlabelled material bromination was complete in 2 - 3 hr, whereas azoxymethane of high specific activity always required a 5 - 6 hr reaction time (4 experiments). Although it is tempting to propose an isotope effect to explain these results, further work is necessary to substantiate such a possibility.

Miligram quantities of MAM-acetate (5) were purified by thin layer chromatography (TLC) and visualized under UV light. The MAM-acetate band was scraped and eluted with methylene chloride. Caution should be exercised during solvent evaporation so as not to lose product. TLC-purified ^{14}C-MAM-acetate was analyzed by HPLC under conditions markedly different than those reported by Fiala et al.[11] Neither HPLC methodologies may be utilized for preparative purification owing to difficult product recovery from the eluting solvent (approx. 12% $MeOH/H_2O$). Long range spin coupling (J=1.46) between $N-CH_3$ and NCH_2 functions confirmed the structural assignment.

Assembly for the Micro-scale Preparation of Azoxymethane



a. 25 ml three necked flask. b. 14/20 "Thread-Tite" joint. c. "Thread-Tite" teflon/silicon septum cap. d. 10 ml bulb long necked trap with side arm and including a gas bubbler that extends close to the bottom of trap. e. 10 ml bulb filled with anhydrous $CaCl_2$. f. Gas inlet tube extending close to the bottom of 3 necked flask. g. 12/5 ball and socket joint secured with a clamp. h. gas outlet tube secured to reaction flask. i. dewar flask j. ice bath k. scintillation vial with 10 ml cocktail and a pipet bubbler.

EXPERIMENTAL SECTION

NMR data were obtained in 100% $CDCl_3$ using an IBM NR/80 spectrometer. Radioactive disintegrations were measured on a Beckman LS-355 liquid scintillation counter using Amersham PCS or NEN formula 963 as a counting cocktail and dpm were determined using OXI-TEST internal standard from Radiomatic Instrument and Chemical Co Inc. Standard N,N'-dimethylhydrazine dihydrochloride was purchased from Aldrich; m-chloroperbenzoic acid and unlabelled MAM-acetate were purchased from Sigma. TLC plates were silica gel GF, 10 x 20 cm, 250 micron, glass plates purchased from Analtech. HPLC was carried out using a Laboratory Data Control (L.D.C.) Gradient System controlled by a Commodore PCM 1611 control module. The column effluent was monitored using a L.D.C. Spectromonitor III variable wavelength UV detector and the radioactivity was measured by radioactive flow detector FLO-ONE model HP using Flo-Scint II cocktail purchased from Radiomatic Instrument and Chemical Co. The column was a L.D.C. Excalibar Spherisorb ODS 5μ, 4.6 x 250 mm. All glassware utilized had "Thread-Tite" 14/20 joints and caps purchased from Reliance Glass Work, Inc.

N,N'-[Methyl-^{14}C]-dimethylhydrazine dihydrochloride (1):

Dimethylhydrazine (1), having a specific activity of 112.5 mCi/mmol, was prepared in 50% yield by the methed of Kumar et al. [10] The purity of the compound was determined by comparing its TLC with that of standard N,N'-dimethylhydrazine dihydrochloride.

^{14}C-Azomethane (2) and ^{14}C-Azoxymethane (3):

These compounds were prepared in sequence without separating 2. Thus, in a 25 ml 3-necked round bottom flask fitted with a teflon/silicon rubber septum and a gas inlet tube, was placed 223 mg of Amberlite IRA-93 previously washed 4-5 times with water. Yellow mercuric oxide 56 mg, (0.26 mM) and 1 ml of water was added. Two cooled traps each containing 10 ml of methylene chloride were utilized. To trap #1 was added 50 mg of m-chloroperbenzoic acid. Trap #1 was cooled to approx. 10°C with cold water and trap #2 was cooled in an ice-water bath (approx. 0°C). The reaction flask was cooled in an ice bath and 24.36 mg, (0.178 mM, 20 mCi, 112.5 mCi/mmol) of N,N'-[methyl-^{14}C]-dimethylhydrazine dihydrochloride (1) dissolved in 1 ml of water was added by syringe through the septum. The ice bath was removed after 30 minutes and the reaction mixture was stirred at room temperature for 2 hours. Argon was then bubbled through the reaction

mixture at a flow rate of 5-6 ml/min and the temperature of the oil bath was gradually raised from room temperature to 80-85°C. The reaction was stirred for an additional 2-3 hours at this temperature. Heating and gas flow were stopped and the solutions from the two traps were combined and stored at -4°C for 24 hrs. The solution was brought to room temperature and passed over basic alumina (2 gm) using a 1 x 15 cm column. The column was washed with methylene chloride (5 ml) and the eluent was distilled at 70°C using a 15 cm vacuum jacketed vigreaux column. The total radioactivity in the residue was 16 mCi. The yield of ^{14}C-azoxymethane (3) was 80% based on 1 (NMR of the residue from unlabelled 3 exhibited NMR ($CDCl_3$) δ 3.18 (bs, 3H,=NCH_3), 4.05 (bs, 3H, CH_3 -NO=).

^{14}C-Bromoazoxymethane (4) was prepared by placing 16 mCi (0.145 mM) of 3, 70 mg (0.39 mM) of N-bromosuccinimide and 3 ml of carbon tetrachloride in a 10 ml pear-shaped flask fitted with a water condenser protected by a calcium chloride tube. The mixture was stirred at 50-55°C under a 60 watt lamp held at a distance of 2 cm. Development of a light orange color in the mixture indicated reaction completion (5-6 hrs.). The mixture was cooled to room temperature and filtered through a disposable pasteur pipet plugged with glass wool. The filtrate containing 4 was immediately converted to ^{14}C-MAM-acetate without further purification.

^{14}C-Methylazoxymethanol acetate (5): To the solution of 4 in carbon tetrachloride was added 85 mg (0.50 mM) of silver acetate. The mixture was protected from light, stirred at room temperature overnight, and filtered using a pasteur pipet plugged with glass-wool. The solvent was removed at 77°C using a short path distillation head affording a residue containing 10 mCi ^{14}C. The crude product was purified (TLC) on silica-gel using ethyl acetate:hexane (1:2) as eluting solvent. The band which chromatographed with unlabelled standard 5 was scraped, eluted with methylene chloride, and evaporated on a rotary evaporator at room temperature (caution must be exercised to avoid loss of 5) to afford 4.03 mCi (20.1% yield) of pure 5 exhibiting a specific activity of 110.3 mCi/mmol (determined from a weighed aliquot). The chemical and radiochemical purity of TLC-purified 5 was analyzed by HPLC. The only detectable contaminant was 3 (2.8%). TLC purified 5 was eluted using a 5-15% methanol linear gradient at 0.8 ml/min over 67 minutes. Compounds 3 and 5 were detected by monitoring both absorbance at 235 nm (0.05 AUFS) and dpm at 85% static efficiency using a 3:1 cocktail to eluent ratio and a 0.5 ml flow cell.

Retention times were 19.4 and 33 min. for 3 and 5, respectively. NMR ($CDCl_3$) for both labelled and unlabelled standard exhibited δ 2.15 (s, 3H, CH_3CO), 4.06 (t, 3H, J = 1.46 Hz, CH_3NO) 5.37 (q, 2H, J=1.46 Hz,=NCH_2O). The distillate from crude 5 contained 6 mCi of 3. This distillate on rebromination, acetylation and purification by TLC afforded 900 μCi of pure 5. The total yield of 5 was 4.93 mCi (24%) based on 1.

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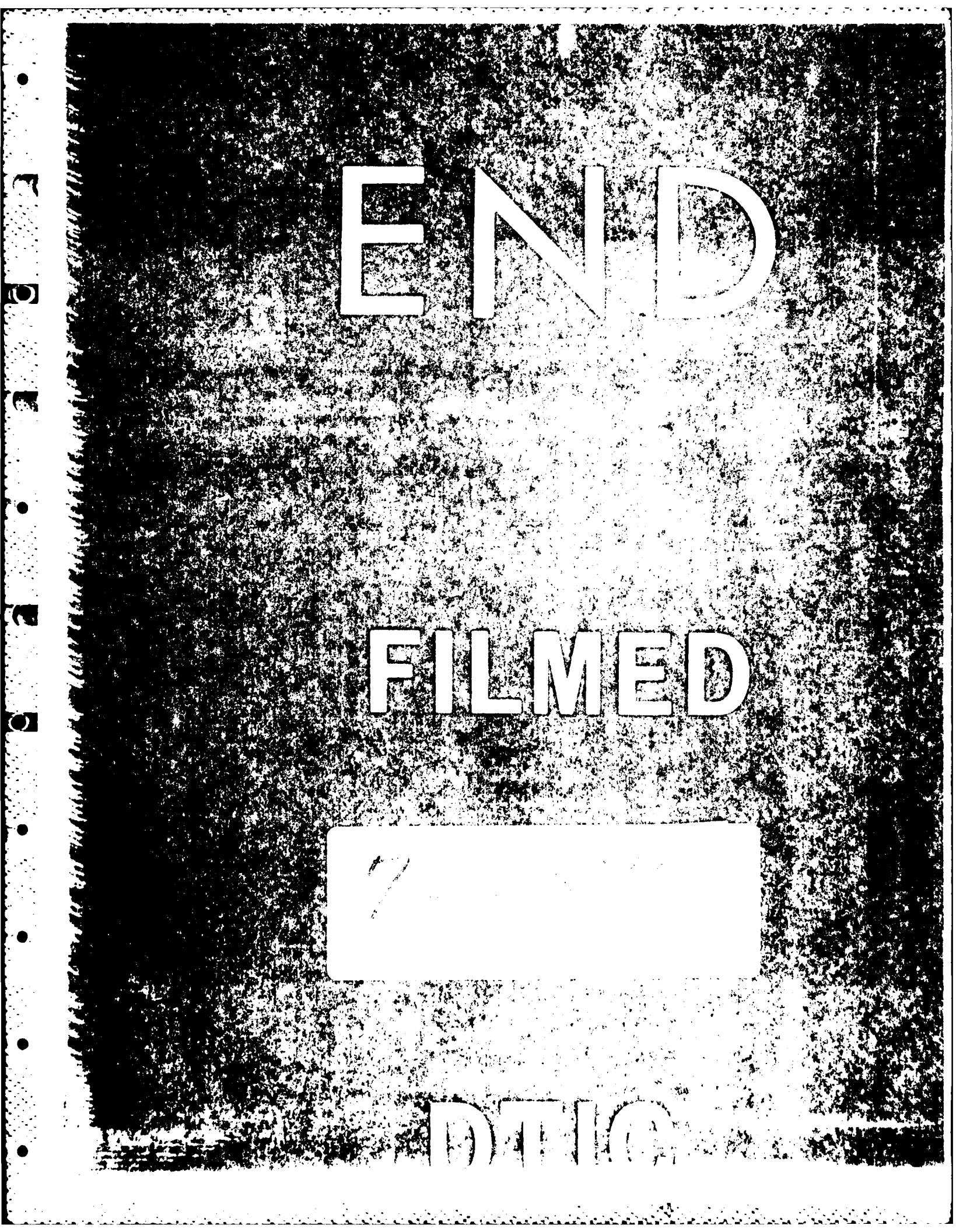
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